## Supporting Information

# Stylissadines A and B – The First Tetrameric Pyrrole-Imidazole Alkaloids

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**Extraction and isolation**

The sponge *Stylissa caribica* was collected by scuba at Little San Salvador in the Bahamas (74 ft depth, July 2000). The samples were immediately frozen after collection and kept at -20 °C until extraction. The freeze dried sponge samples of *Stylissa caribica* (94.7 g) were crushed with a mill and extracted at RT exhaustively in a 1:1 mixture of $CH_2Cl_2$/MeOH. The orange-colored crude extract of *Stylissa caribica* was partitioned between *n*-hexane (4 x 400 mL) and MeOH (300 mL). The MeOH extract was then partitioned between *n*-BuOH (3 x 500 mL) and $H_2O$ (300 mL). The resulting *n*-BuOH (15.9 mg) phase from the solvent partitioning scheme was purified by gel chromatography on Sephadex LH-20 (Pharmacia) using MeOH as mobile phase. Final purification of the isolated compounds was achieved by preparative $RP_{18}$ HPLC on a Kromasil $RP_{18}$ column (16 x 250 mm, 10 µm) applying a MeCN/TFA (0.1% in water) gradient.

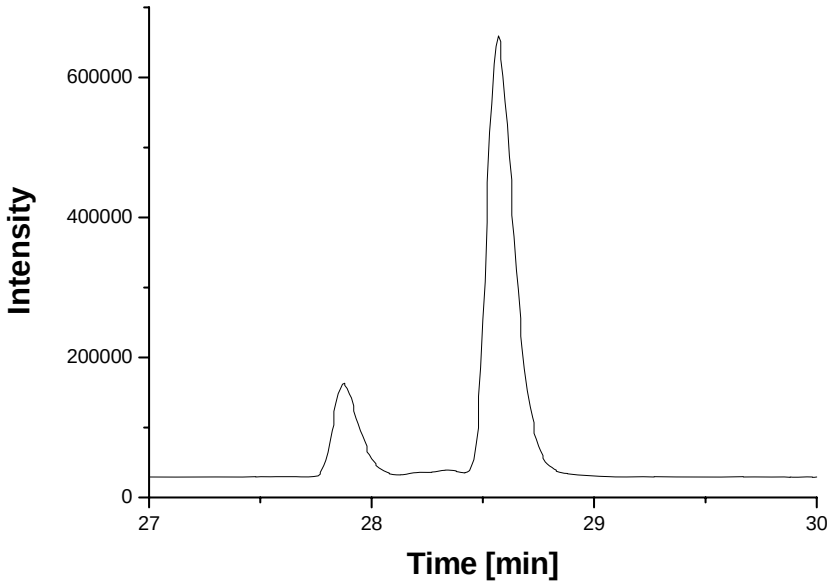



**Figure S1:** Section of the HPLC chromatogram of a LH-20 fraction of *Stylissa caribica* including the stylissadines A (**5**) and B (**6**).

*Detection by light scattering detector; Kromasil $RP_{18}$, 5 µm, 4.6 x 250 mm, 1 ml/min*
*Gradient: 40 min 0-80% B/A – 45 min 100% B – 50 min 50% B/C – 55 min 100% C*
*(A: $H_2O$ + 0.1% TFA, B: MeCN + 0.1% TFA, C: MeOH)*

**MS analysis**

High resolution mass spectra of stylissadine A (**5**) und B (**6**) were recorded on an oTOF mass spectrometer equipped with an ESI source in positive mode (microTOF$_{LC}$, Bruker Daltonics). For fragmentation API-CID-MS/MS was applied. Figure S2 (top) shows the API-CID-MS/MS spectrum of stylissadine B (**6**). The isotopic cluster at *m*/*z* = 1639 is the $[M+H]^+$ precursor ion of **6**. The loss of two guanidine groups (*m*/*z* = 59) from the precursor ion and the typical fragment (*m*/*z* = 252) for the dibromopyrrolecarboxylic acid moiety are observed. Figure S2 (bottom) shows the HR-MS spectrum of stylissadine A (**5**). For calculation of the molecular formula the doubly charged peak was used because of a better mass accuracy in this mass division. Ion transfer and TOF settings were optimized for masses under *m*/*z* 1000. The mass spectrometer was calibrated using a solution of sodium formiate clusters.

Figure S3 (three sections, a to c) shows the API-CID-MS/MS spectra of stylissadine B (**6**) in comparison to massadine (**4**). At *m*/*z* = 828.8 the $[M+H]^+$ ion of massadine appeared. Starting from this mass, the fragmentation of stylissadine B (**6**) and massadine (**4**) is identical. In MS/MS spectra of stylissadine B (**6**) $[M+H]^+$ at *m*/*z* = 1639 is precursor ion, while in MS/MS spectra of massadine (**4**) $[M+H]^+$ at *m*/*z* = 828.8 is precursor ion.

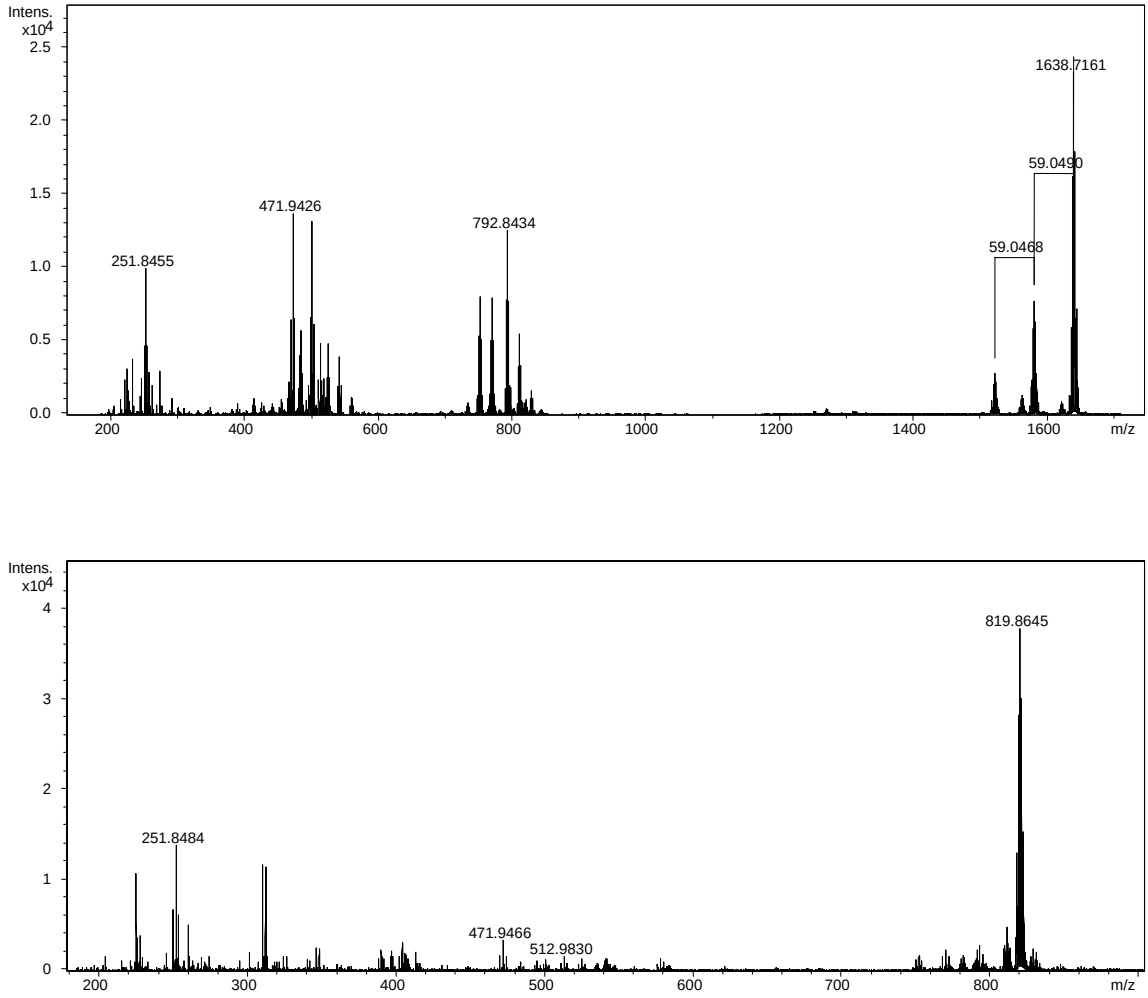



**Figure S2:** MS spectra of stylissadines: (top) API-CID-MS/MS spectrum (ESI, positive mode) of stylissadine B (**6**) *m*/*z* 1638.7 $[M+H]^+$; (bottom) HR-MS spectrum (ESI, positive mode) of stylissadine A (**5**) *m*/*z* 819.9 $[M+2H]^{2+}$.

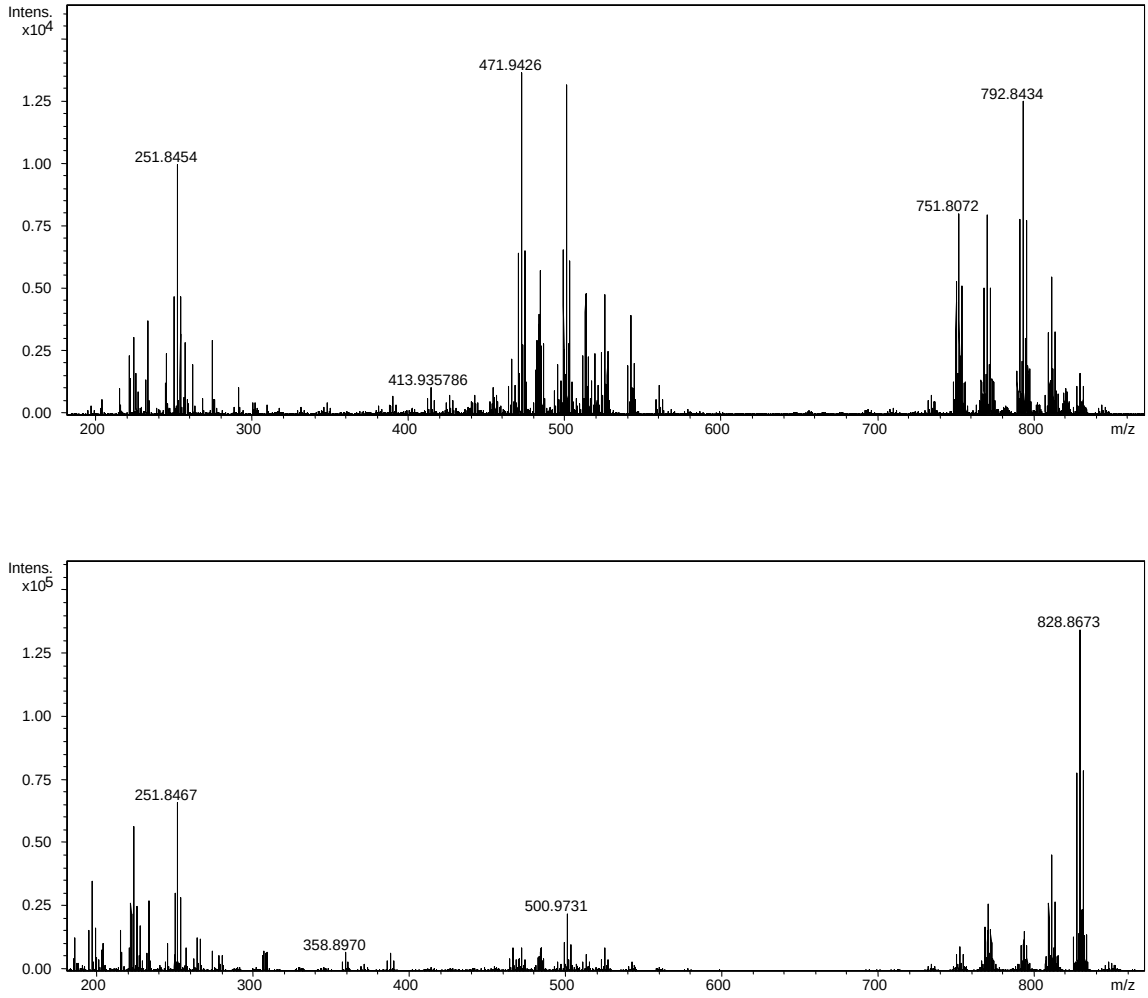



**Figure S3a:** Section 1 from *m*/*z* 200 to *m*/*z* 860 of the API-CID-MS/MS spectrum (ESI, positive mode) of stylissadine B (**6**, top) and massadine (**4**, bottom).

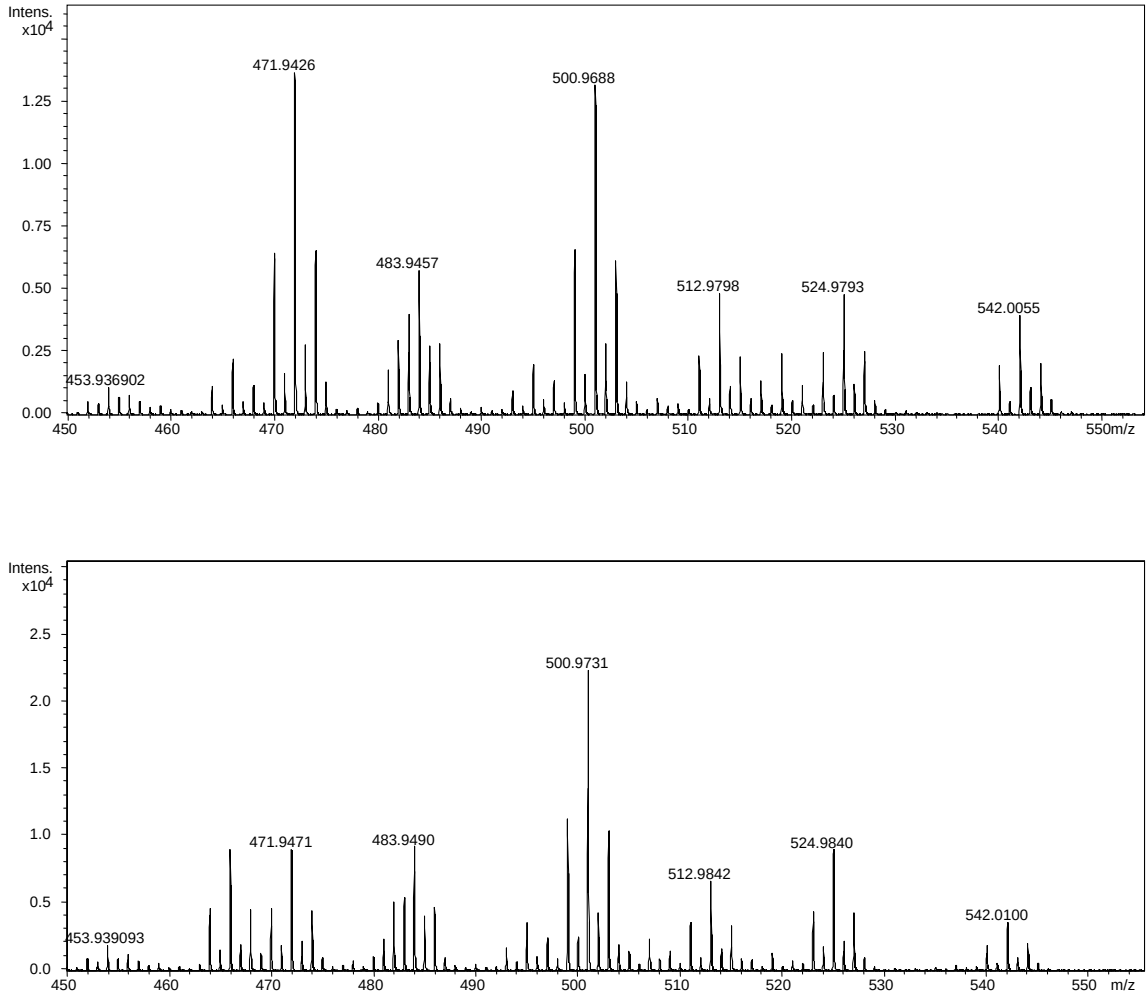



**Figure S3b:** Section 2 from *m*/*z* 450 to *m*/*z* 550 of the API-CID-MS/MS spectrum (ESI, positive mode) of stylissadine B (**6**, top) and massadine (**4**, bottom).

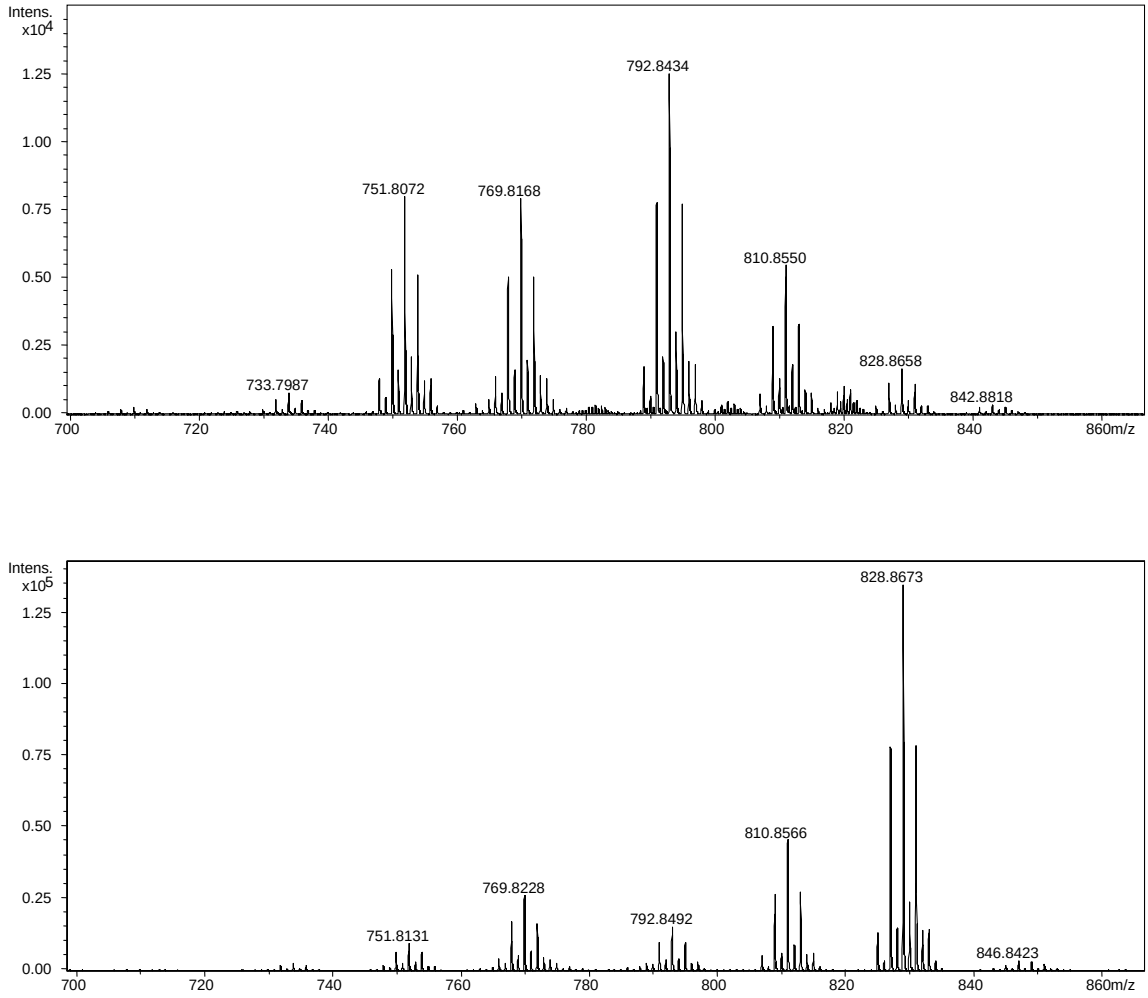



**Figure S3c:** Section 3 from *m*/*z* 700 to *m*/*z* 870 of the API-CID-MS/MS spectrum (ESI, positive mode) of stylissadine B (**6**, top) and massadine (**4**, bottom).

## NMR analysis

**Table S1.** NMR-spectroscopical data for the two tetracyclic substructures of stylissadine A (**5**) in DMSO-$d_6$.[a]

| Pos.[b] | δ($^{13}$C) | δ($^{1}$H) (mult, *J* in Hz) | δ($^{15}$N)[c] | COSY | HMBC |
|---|---|---|---|---|---|
| 1 | 40.8 | 2.12 (m) | | 1a, 2, 15 | 2, 15 |
| 2 | 45.3 | 2.22 (d, 11.7) | | 1 | 1, 1a, 3, 9, 13 |
| 3 | 86.8 | | | | |
| 3-OH | | 7.76 (s) | | 7 | 2, 3, 13 |
| 4 | | 9.44 (s) | 106 | | 3, 5, 7 |
| 5 | 157.1 | | | | |
| 5-$NH_2$ | | 8.22 (br s) | 75 | | |
| 6 | | 9.26 (s) | 100[d] | | |
| 7 | 89.8 | 5.37 (d, 2.2) | | 3-OH, 6 | 3, 5, 9 |
| 9 | 81.9 | 5.46 (s) | | 10 | 7, 11, 14 |
| 10 | | 9.26 (s) | 96[d] | | |
| 11 | 157.3 | | | | |
| 11-$NH_2$ | | 8.22 (br s) | 75 | | |
| 12 | | 9.17 (s) | 90 | | 9, 11, 13 |
| 13 | 69.1 | | | | |
| 14 | 81.6 | 3.49 (s) | | 15 | 1, 1b, 2, 9, 13, 15 |
| 15 | 47.9 | 2.00 (m) | | 1, 1b, 14 | 1a, 14 |

[a] Chemical shifts δ are given in [ppm]. $^{1}$H and $^{13}$C chemical shifts are referenced to the DMSO-$d_6$ signal (2.50 and 39.5 ppm, respectively).
[b] Since 5 is $C_2$ symmetric, 1 stands for 1 and 1'; 2 for 2 and 2' etc.
[c] $^{15}$N-NMR spectra were not calibrated with an external standard. δ($^{15}$N) has an accuracy of about 1 ppm in reference to $NH_3$ (0 ppm).
[d] Assignment may be interchanged.

**Table S2:** NMR-spectroscopical data for the four dibromopyrrole carboxamide side chains (a=d and b=c) of stylissadine A (**5**) in DMSO-$d_6$.[a]

| Pos.[b] | **a** | | **b** | |
|---|---|---|---|---|
| | $^{13}$C/$^{15}$N | $^{1}$H | $^{13}$C/$^{15}$N | $^{1}$H |
| 1 | 42.6 | 3.68/3.39 | 41.4 | 3.35/3.28 |
| 2 | (107) | 8.08 | (107) | 8.34 |
| 3 | 158.8 | | 159.3 | |
| 4 | 127.7 | | 127.3 | |
| 5 | 112.3 | 6.80 | 112.7 | 6.87 |
| 6 | 97.9 | | 97.9 | |
| 7 | 104.7 | | 104.9 | |
| 8 | (166) | 12.68 | (165) | 12.45 |

[a] Chemical shifts δ are given in [ppm]. $^{1}$H and $^{13}$C chemical shifts are referenced to the DMSO-$d_6$ signal (2.50 and 39.5 ppm, respectively). For positions no. 2 and 8 δ($^{15}$N) is given in parenthesis in the δ($^{13}$C) column. $^{15}$N-NMR spectra were not calibrated with an external standard. The δ value has an accuracy of about 1 ppm in reference to $NH_3$ (0 ppm).
[b] Since **5** is $C_2$ symmetric, side chain a is identical to d and side chain is identical to c.

**Table S3.** NMR-spectroscopical data for the two tetracyclic substructures of stylissadine B (**6**) in DMSO-$d_6$.[a]

| Pos. | $\delta(^{13}C)$[b] | $\delta(^{1}H)$ (mult, *J* in Hz) | $\delta(^{15}N)$[b] | COSY | HMBC |
|---|---|---|---|---|---|
| 1 | 40.9 | 2.08 (m) | | 1a, 2, 15 | 1a, 1b, 2, 3, 15 |
| 2 | 45.8 | 2.16 (m) | | 1 | 1, 1a, 3, 9, 13 |
| 3 | 87.0 | | | | |
| 3-OH | | 7.95 (s) | | | 2, 3, 13 |
| 4 | | 9.301 (s) | 106[c] | | 5, 7, 13 |
| 5 | 157.4 | | | | |
| 5-$NH_2$ | | 8.20 (br s) | 74 | | |
| 6 | | 9.46 (s) | 96 | 7 | 3, 5 |
| 7 | 90.0 | 5.42 (d, 1.8) | | 6 | 3, 5, 9 |
| 9 | 82.4 | 5.98 (s) | | 10 | 7, 11, 14 |
| 10 | | 9.304 (s) | 101[c] | 9 | |
| 11 | 157.3 | | | | |
| 11-$NH_2$ | | 8.32 (br s) | 75 | | |
| 12 | | 9.29 (s) | 90 | | 9, 11 |
| 13 | 69.8 | | | | |
| 14 | 83.7 | 3.78 (s) | | 15 | 1, 1b, 2, 3, 13, 14′, 15 |
| 15 | 50.1 | 1.84 (m) | | 1, 1b, 14 | 1a, 1b, 14 |
| 1′ | 38.8 | 2.21 (m) | | 1d, 2′, 15′ | |
| 2′ | 42.4 | 2.88 (d, 8.4) | | 1′ | 1′, 1d, 3′, 7′, 9′, 13′ |
| 3′ | 84.7 | | | | |
| 3-OH′ | | 7.45 (s) | | | 2′, 3′, 7′, 13′ |
| 4′ | | 9.34 (s) | 113 | | 5′, 7′ |
| 5′ | 156.9 | | | | |
| 5′-$NH_2$ | | 8.32 (br s) | 75 | | |
| 6′ | | 9.39 (s) | 97 | 7′ | 3′, 5′ |
| 7′ | 86.5 | 5.24 (s) | | 6′ | 3′, 5′, 9′ |
| 9′ | 79.2 | 5.61 (s) | | 10′ | 3′, 7′, 11′ |
| 10′ | | 9.24 (s) | 99 | 9′ | 9′, 11′, 13′ |
| 11′ | 156.7 | | | | |
| 11′-$NH_2$ | | 8.43 ( br s) | 75 | | |
| 12′ | | 8.59 (s) | 97 | | 9′, 11′, 13′ |
| 13′ | 68.3 | | | | |
| 14′ | 84.7 | 3.70 (d, 5.1) | | 15′ | 1c, 9′, 13′, 14, 15′ |
| 15′ | 45.7 | 1.94 (m) | | 1′, 1c, 14′ | |

[a] Chemical shifts δ are given in [ppm]. $^{1}H$ and $^{13}C$ chemical shifts are referenced to the DMSO-$d_6$ signal (2.50 and 39.5 ppm, respectively).
[b] $^{15}N$-NMR spectra were not calibrated with an external standard. The δ value has an accuracy of about 1 ppm in reference to $NH_3$ (0 ppm).
[c] Assignment may be interchanged.

**Table S4:** NMR-spectroscopical data for the four dibromopyrrole carboxamide side chains (a to d) of stylissadine B (**6**) in DMSO-$d_6$.[a]

| Pos. | **a** | | **b** | | **c** | | **d** | |
|---|---|---|---|---|---|---|---|---|
| | $^{13}C/^{15}N$ | $^{1}H$ | $^{13}C/^{15}N$ | $^{1}H$ | $^{13}C/^{15}N$ | $^{1}H$ | $^{13}C/^{15}N$ | $^{1}H$ |
| 1 | 43.1 | 3.66/3.11 | 41.2 | 3.57/3.26 | 40.0 | 3.19/3.07 | 39.7 | 3.50/3.35 |
| 2 | (108) | 8.03 | (107) | 8.24 | (105) | 8.12 | (107) | 8.05 |
| 3 | 158.5 | | 159.8 | | 159.5 | | 158.9 | |
| 4 | 127.9 | | 127.5 | | 127.5 | | 127.9 | |
| 5 | 122.2 | 6.75 | 113.0 | 6.91 | 113.1 | 6.81 | 112.5 | 6.82 |
| 6 | 97.8 | | 97.7 | | 97.7 | | 98.1 | |
| 7 | 104.8 | | 105.0 | | 104.3 | | 104.6 | |
| 8 | (166) | 12.71 | (166) | 12.54 | (166) | 12.54 | (165) | 12.38 |

[a] Chemical shifts δ are given in [ppm]. $^{1}H$ and $^{13}C$ chemical shifts are referenced to the DMSO-$d_6$ signal (2.50 and 39.5 ppm, respectively). For positions no. 2 and 8 δ($^{15}N$) is given in parenthesis in the δ($^{13}C$) column. $^{15}N$-NMR spectra were not calibrated with an external standard. The δ value has an accuracy of about 1 ppm in reference to $NH_3$ (0 ppm).

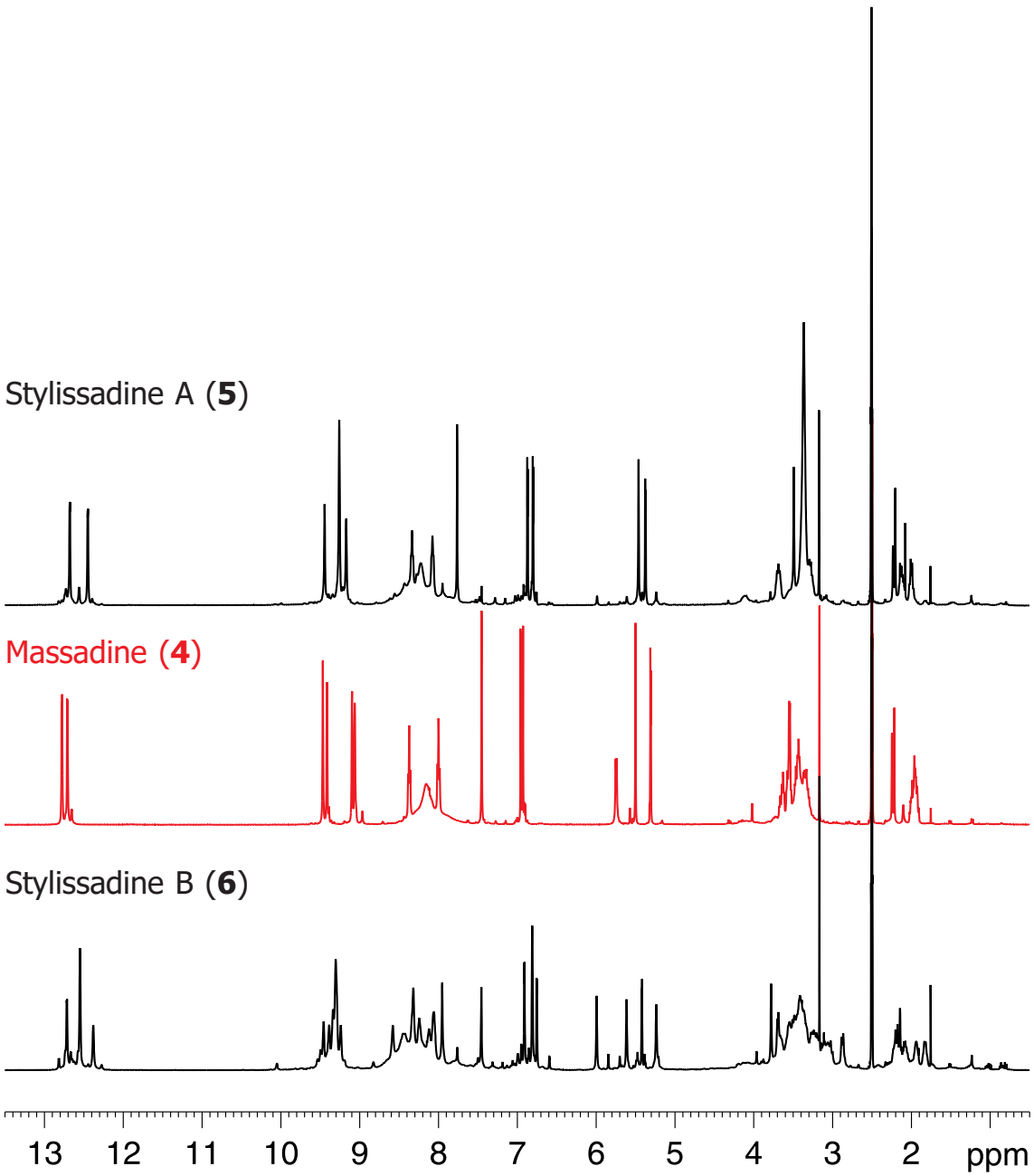



**Figure S4:** 1D $^{1}$H-NMR spectra of stylissadine A (**5**), massadine (**4**), and stylissadine B (**6**) in DMSO-$d_6$. The spectra were recorded on a 400 MHz NMR spectrometer with 16 scans each.

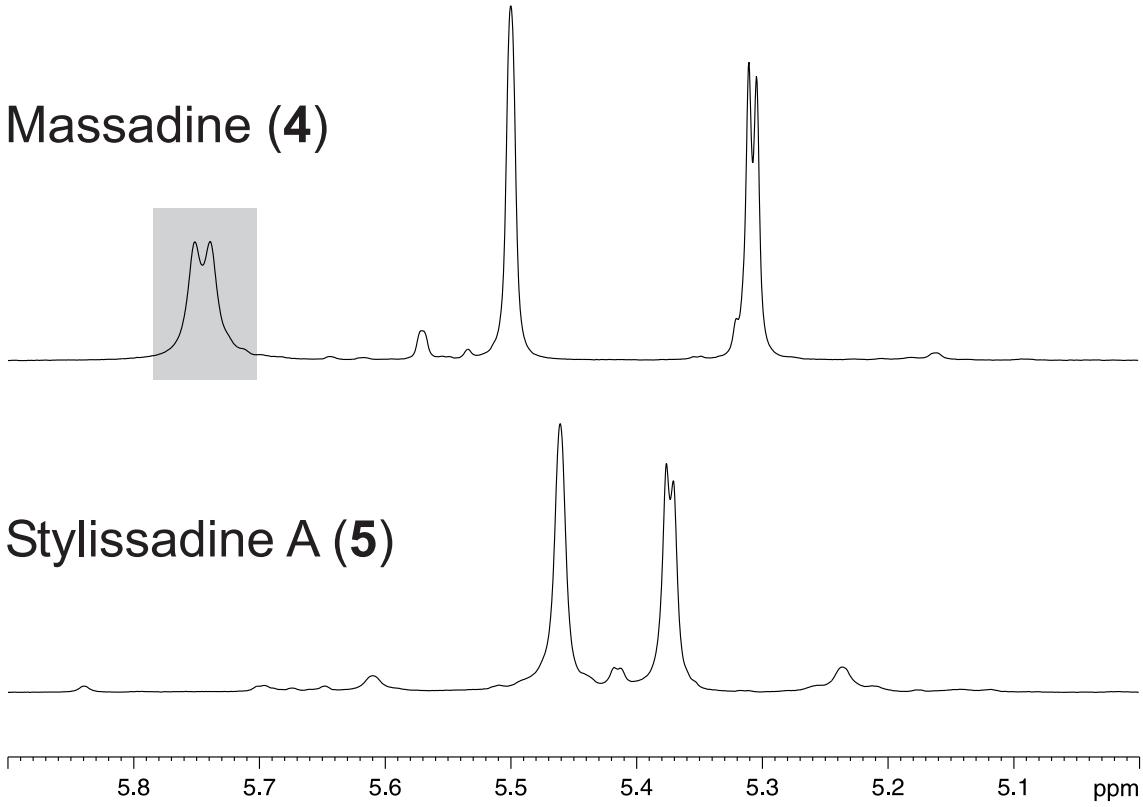



**Figure S5:** Part of the 1D $^{1}$H-NMR spectra of massadine (**4**) and stylissadine A (**5**) in DMSO-$d_6$. The spectra were recorded on a 400 MHz NMR spectrometer with 16 scans each.
The missing signal for the 14-OH group (5.75 ppm) in the spectrum of **5** indicates the ether bridge between the two massadine units.

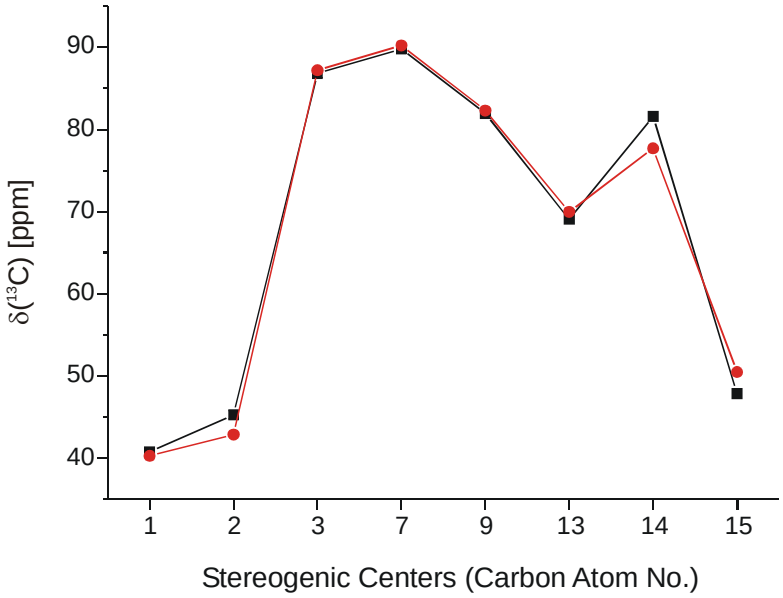
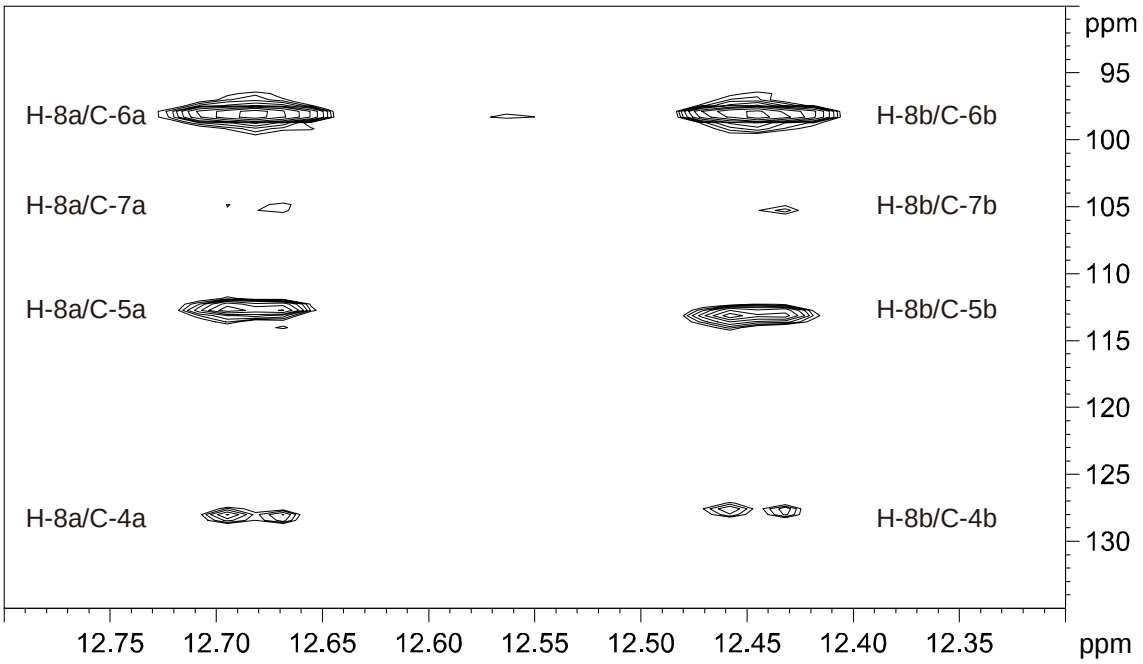



**Figure S6.** Comparison of the $^{13}$C chemical shifts of the eight stereogenic centers of massadine (**4**) to stylissadine A (**5**). The values for massadine (**4**) are represented as red circles, stylissadine A (**5**) as black squares.



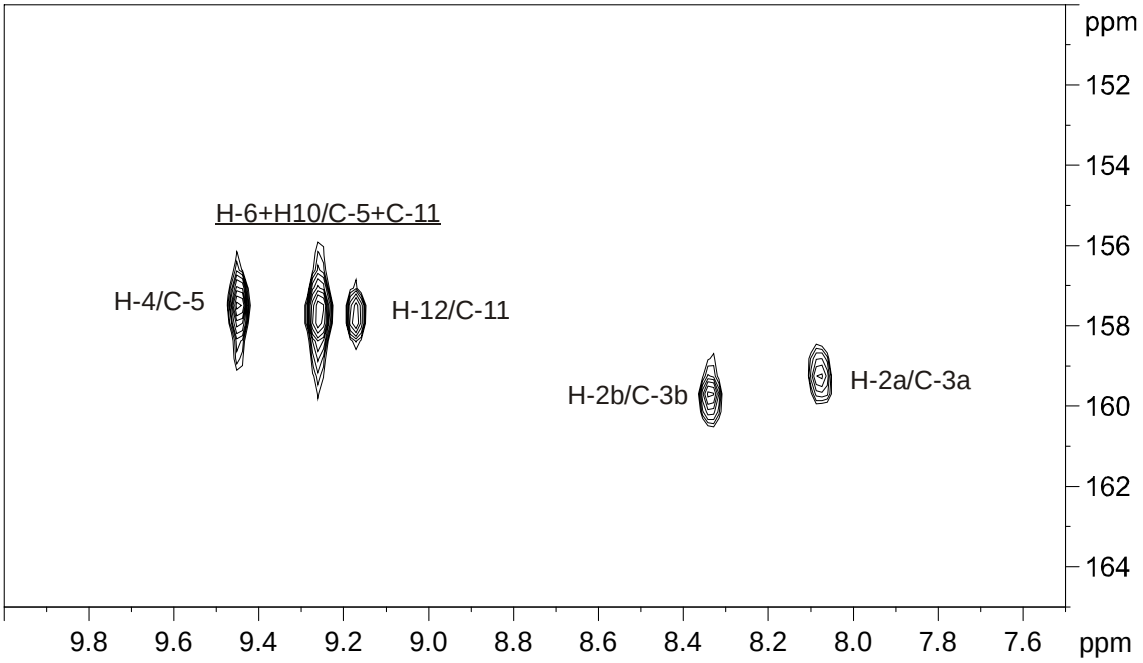




**Figure S7a.** Sections 1 and 2 of the 400 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine A (**5**).

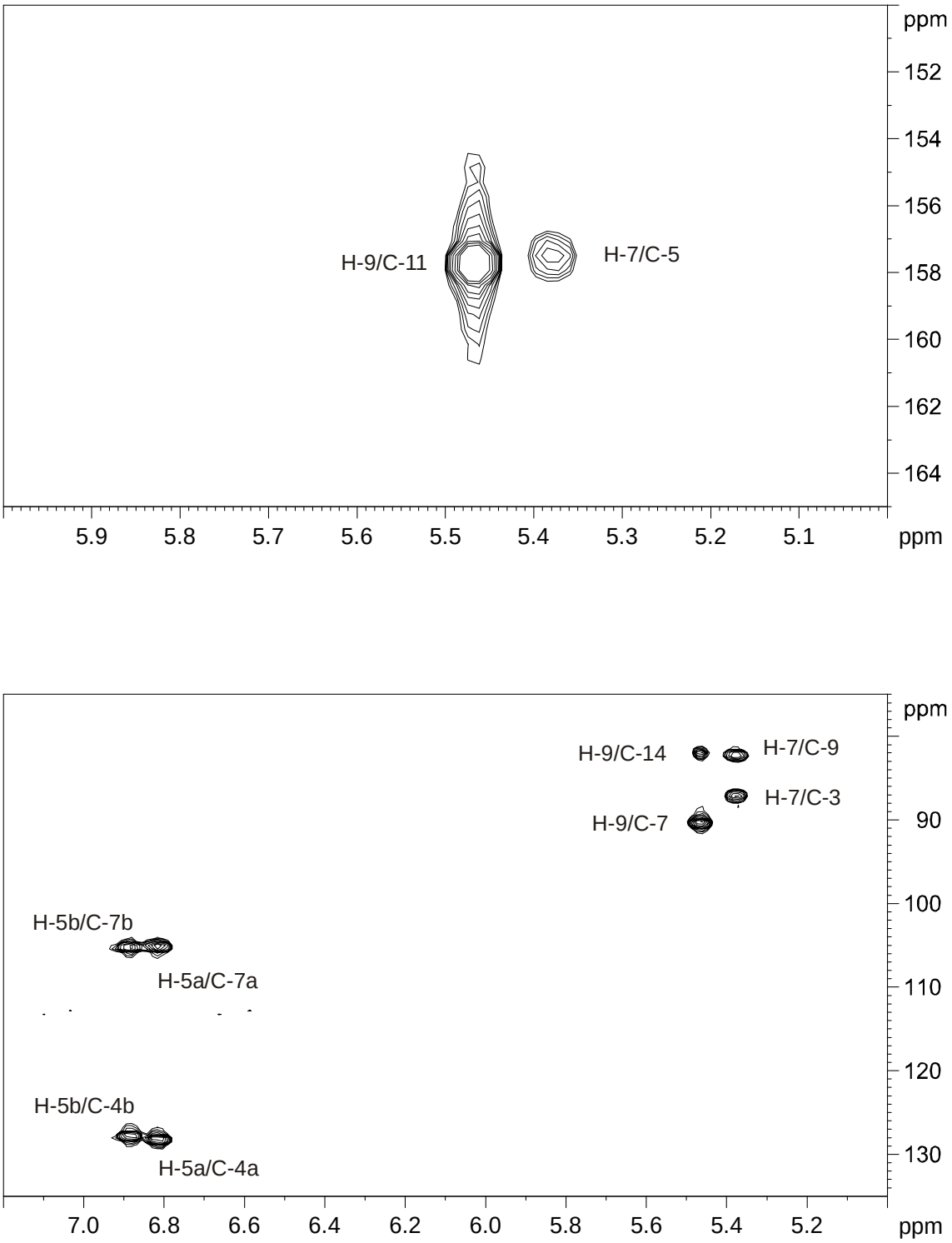



**Figure S7b.** Sections 3 and 4 of the 400 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine A (**5**).

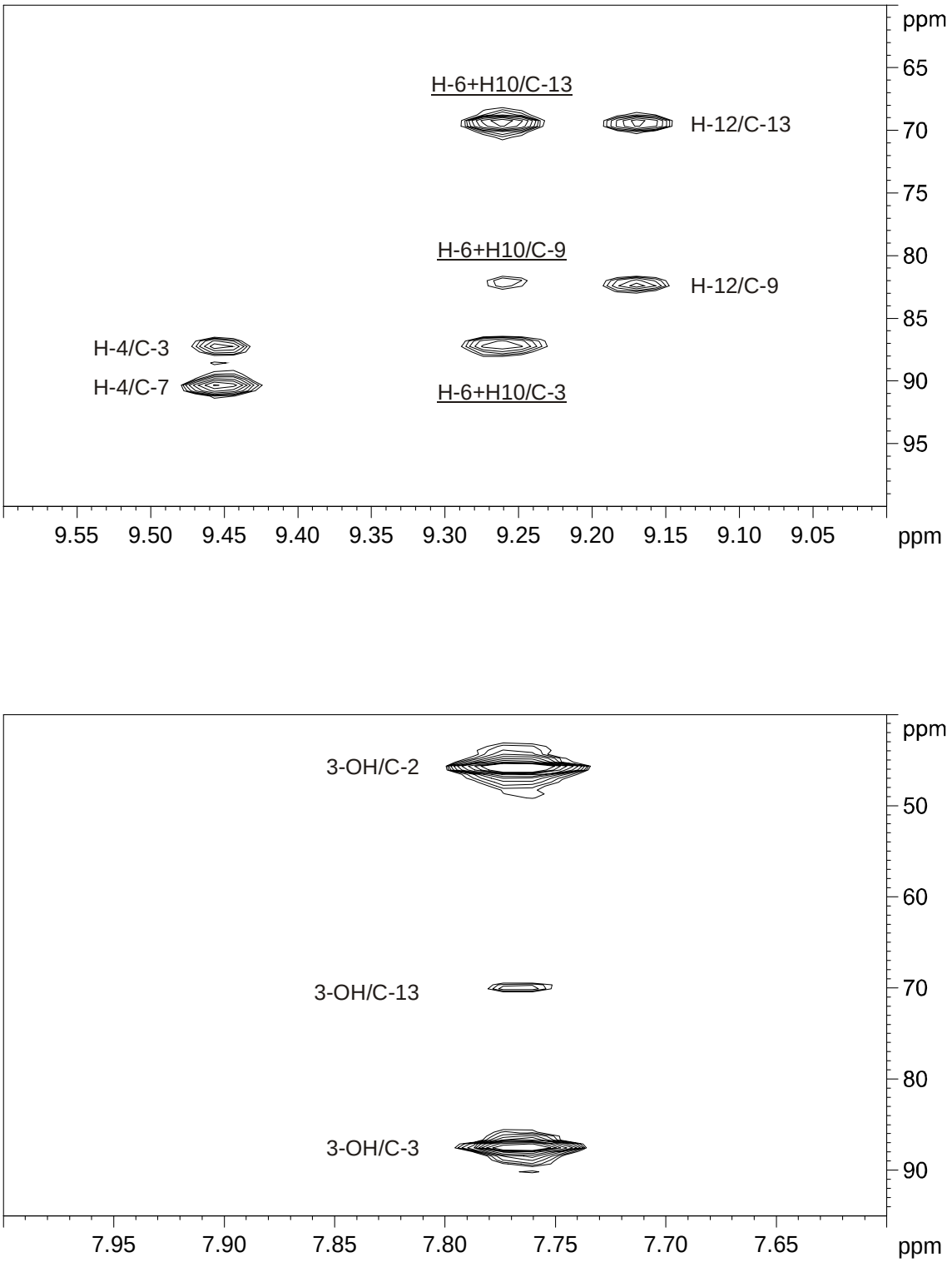



**Figure S7c.** Sections 5 and 6 of the 400 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine A (**5**).

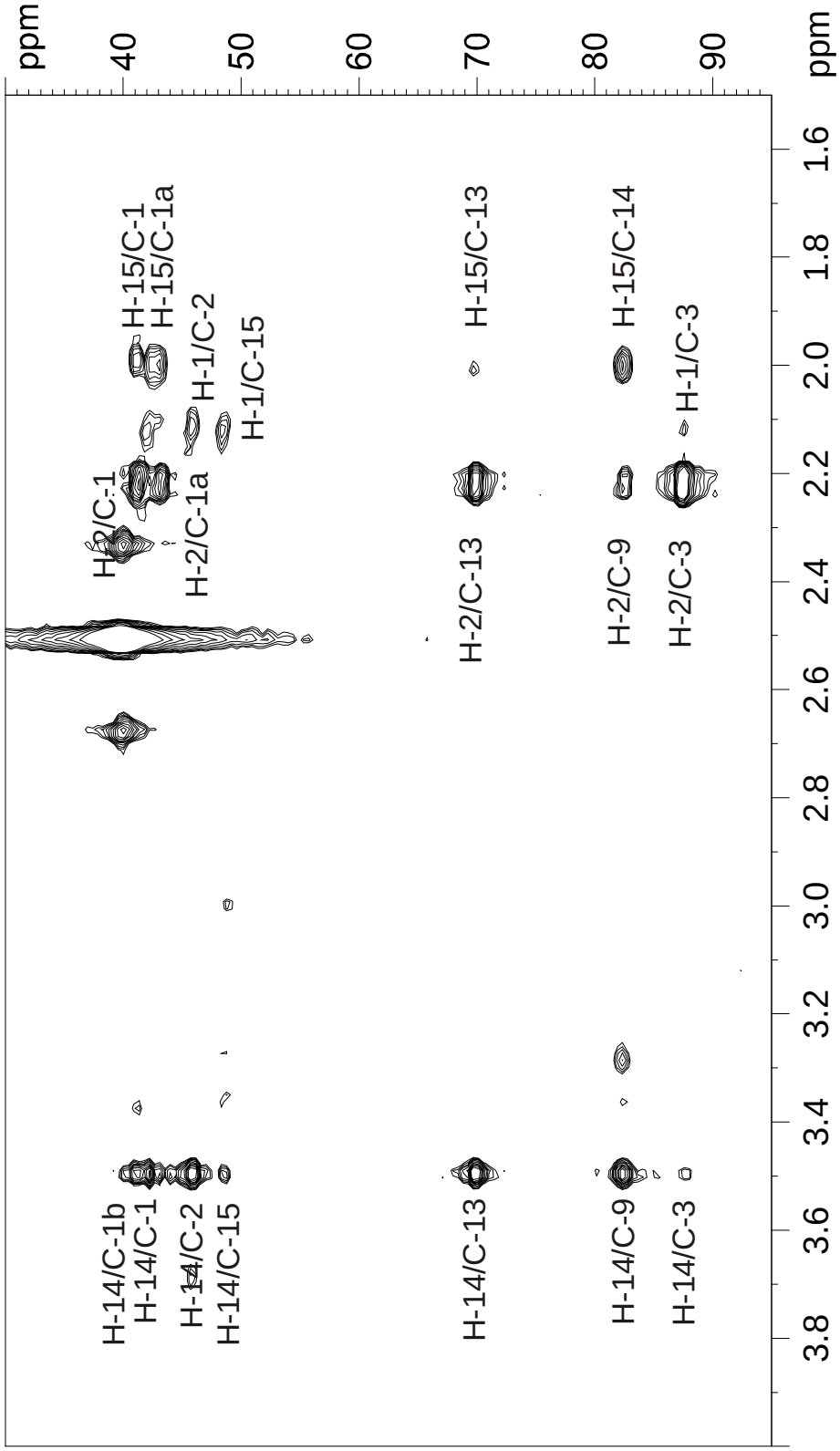
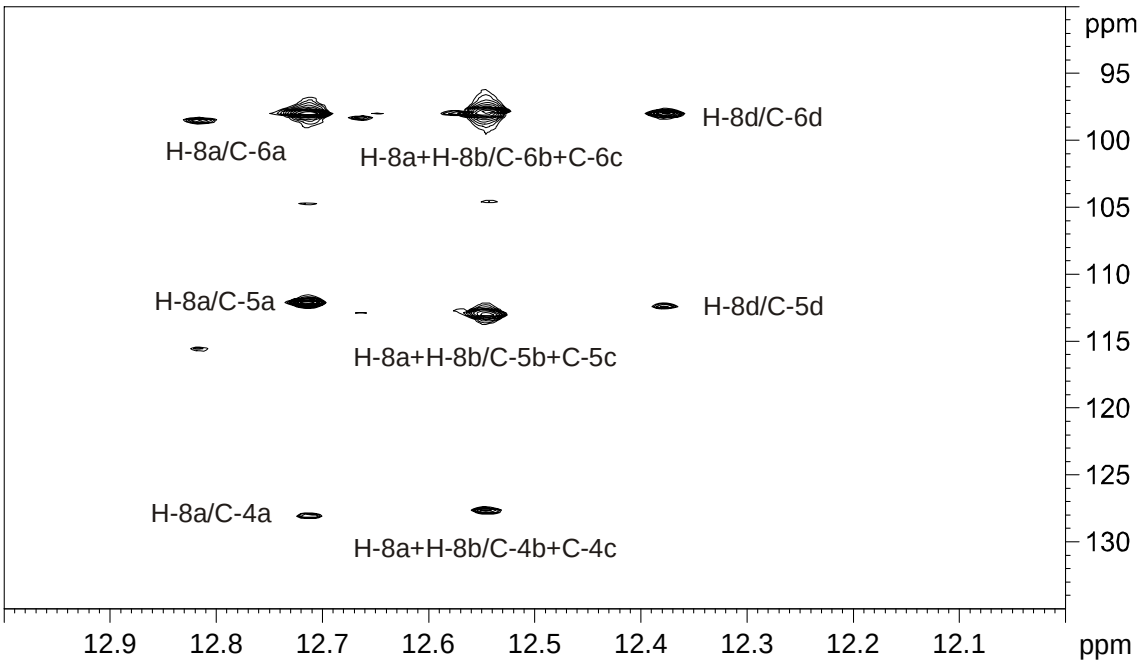



**Figure S7d.** Section 7 of the 400 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine A (**5**).



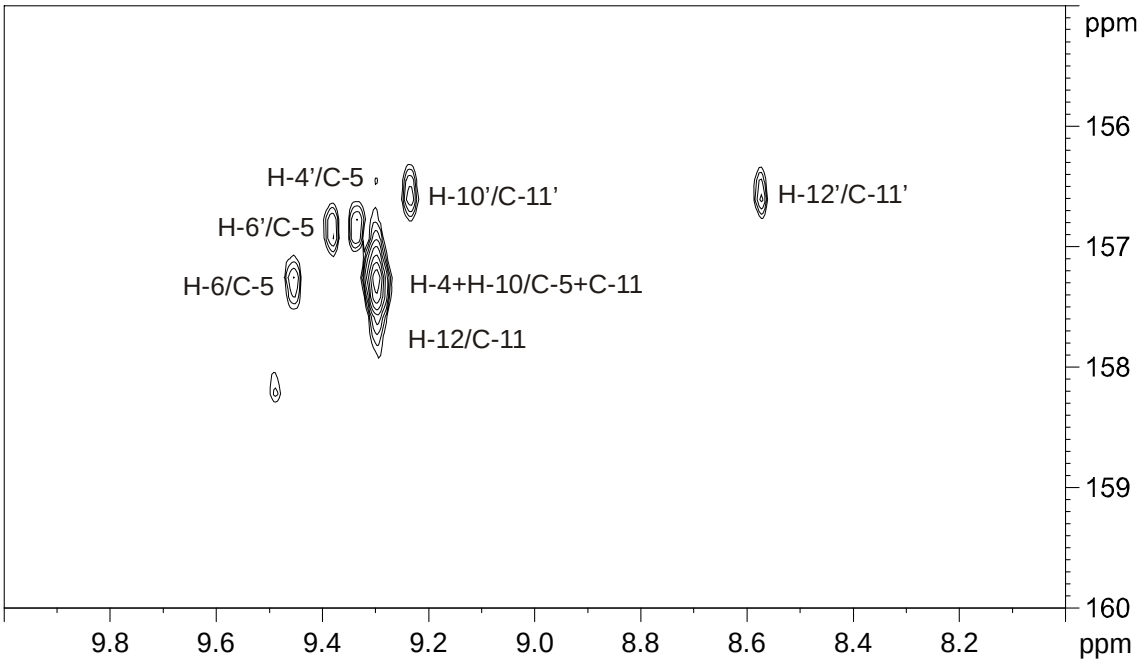




**Figure S8a.** Sections 1 and 2 of the 700 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine B (**6**).

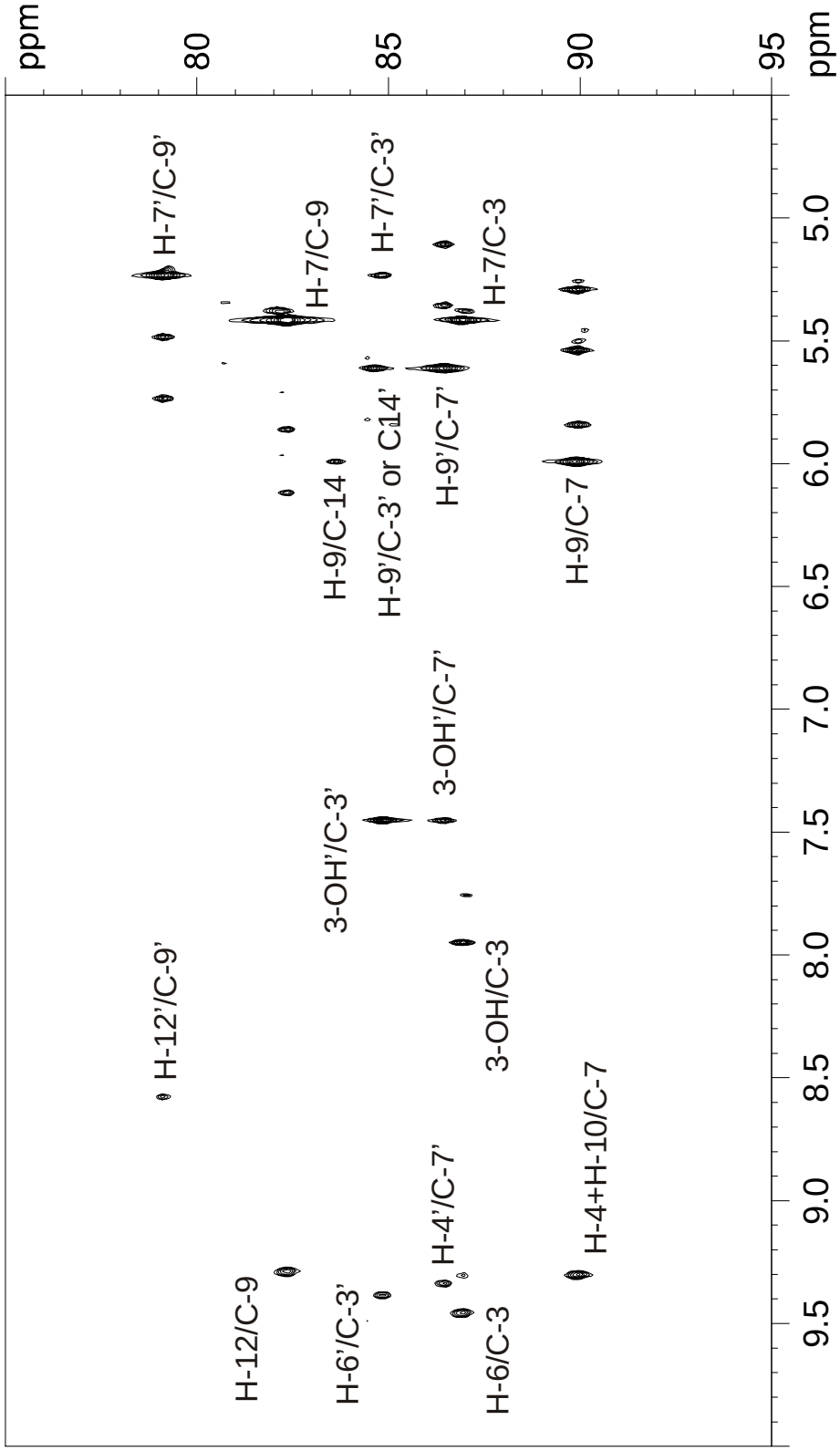



**Figure S8b.** Section 3 of the 700 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine B (**6**).

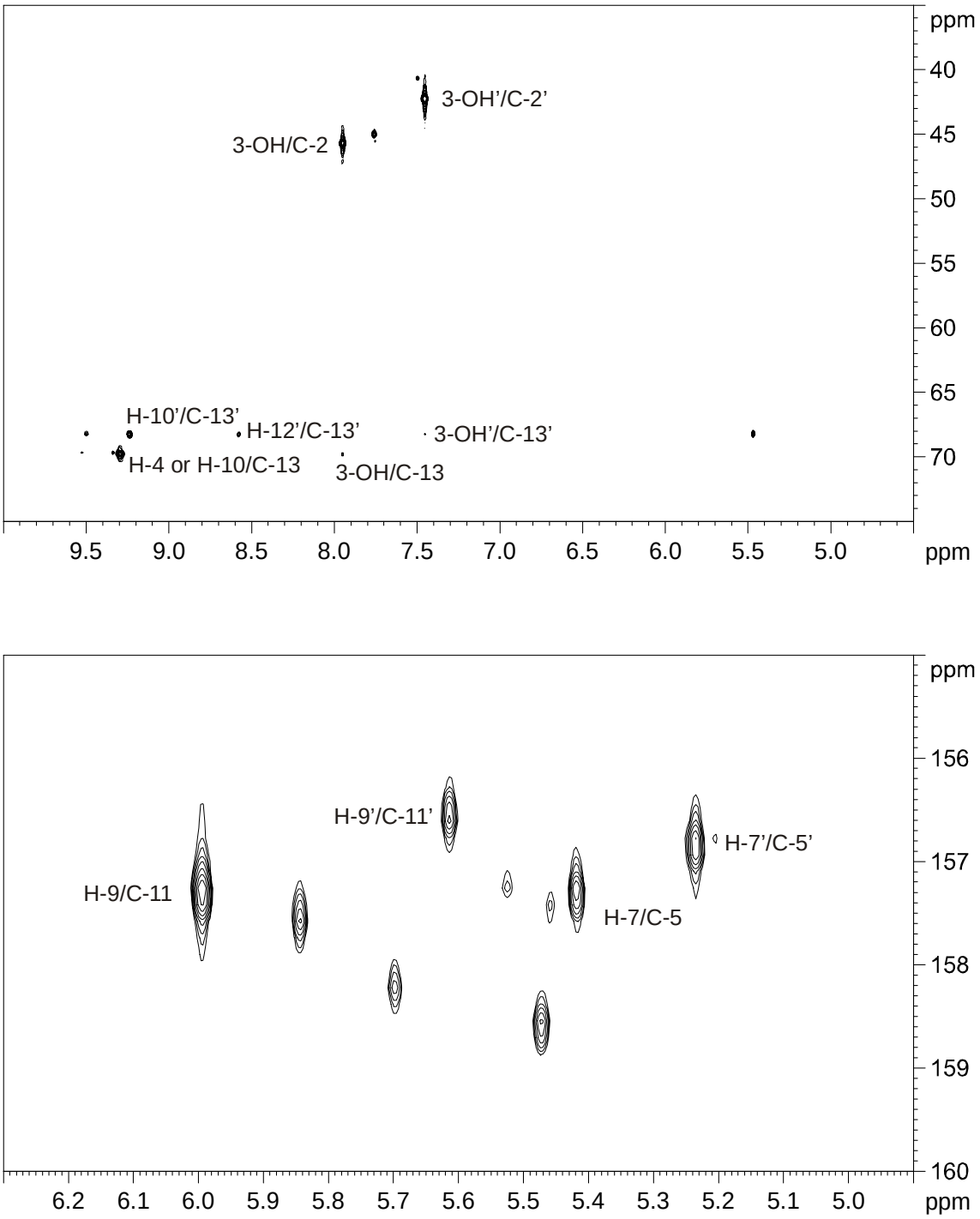



**Figure S8c.** Sections 4 and 5 of the 700 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine B (**6**).

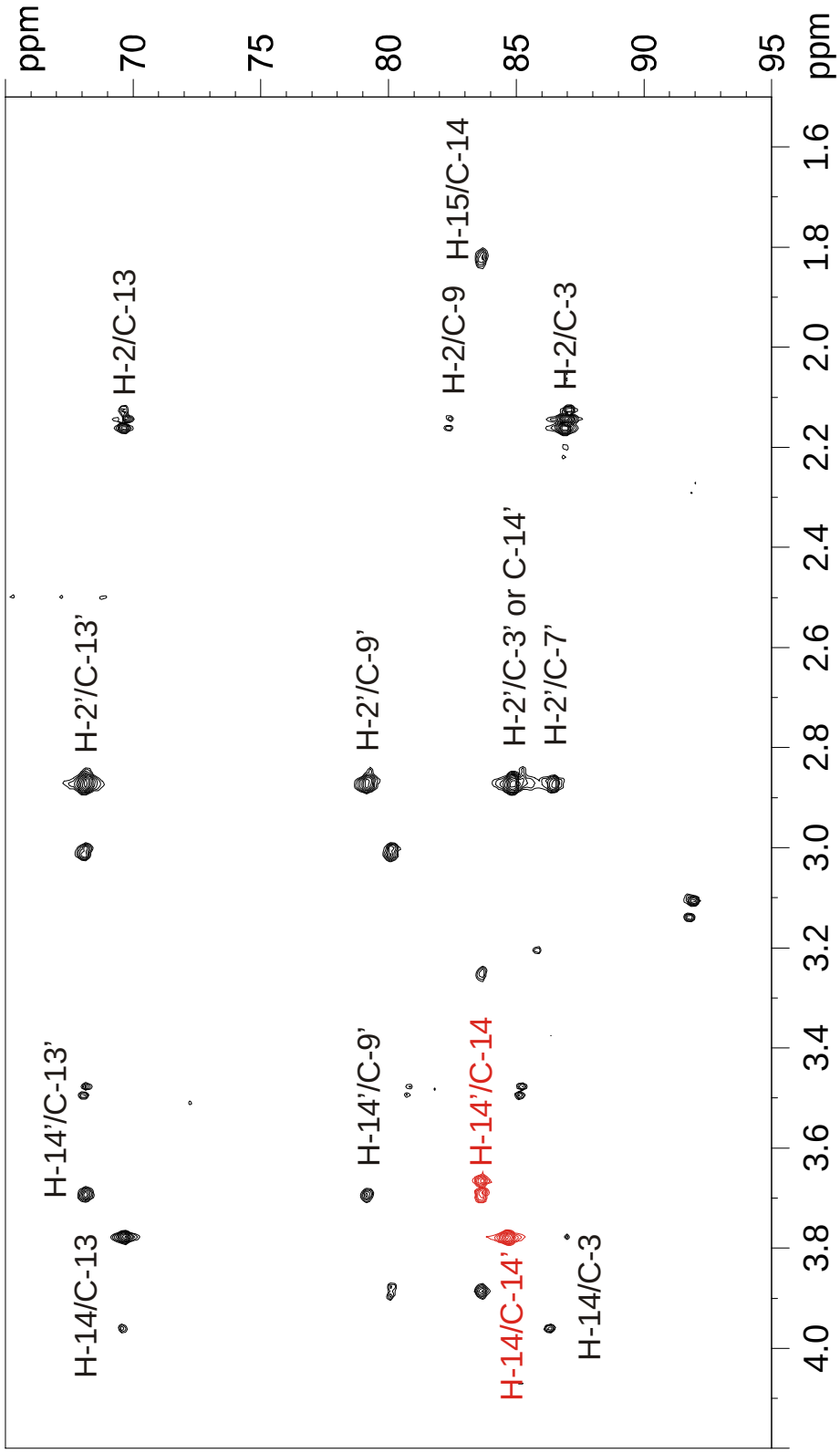
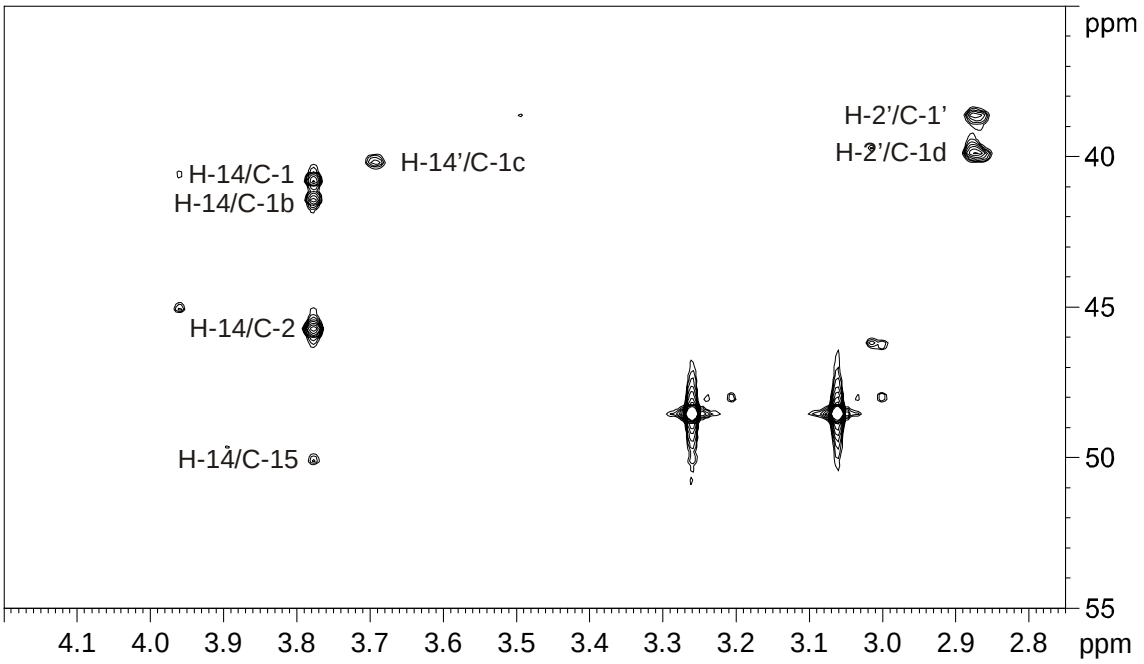



**Figure S8d.** Section 6 of the 700 MHz $^{1}$H,$^{13}$C-HMBC spectrum of stylissadine B (**6**).



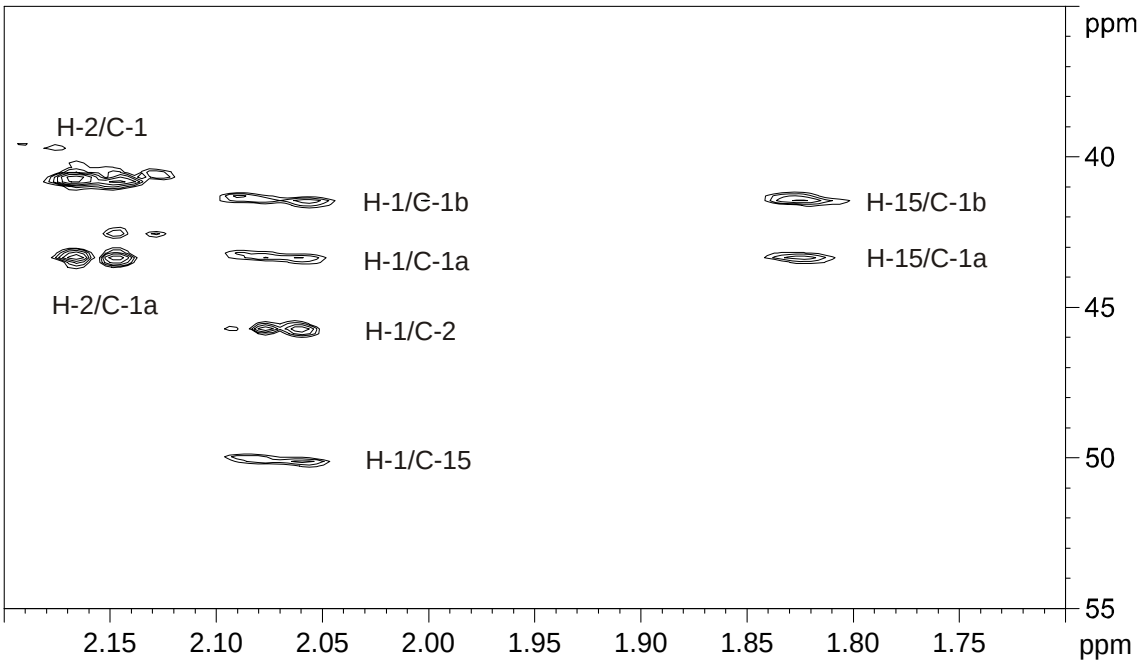




**Figure S8e.** Sections 7 and 8 of the 700 MHz $^{1}H,^{13}C$-HMBC spectrum of stylissadine B (**6**).

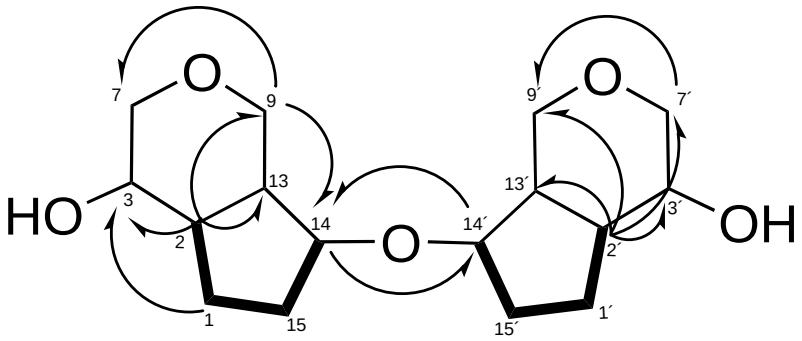



**Figure S9.** Part of the stylissadine structure. The bold printed bonds are defined by COSY correlations observed for stylissadine B (**6**). The arrows indicate the observed HMBC correlations for stylissadine B (**6**).

## Integration of ROESY spectra

Interproton distances for stylissadine A (**5**) and stylissadine B (**6**) were obtained from ROESY spectra with different mixing times (150 and 200 ms). After volume integration of all cross peaks in the ROESY spectra, the intensity data were calibrated using the two geminal protons at C-1a to C-1d (178 pm). Each ROESY spectrum was analysed separately under the assumption of the linear approximation. Since the distances for the different mixing times were identical the assumption is valid.

## Biological Assays

Stylissadines A (**5**) and B (**6**) were tested against gram-negative bacteria (*Eschericha coli* tolC, *Klebsiella pneumoniae*, *Pseudomonas aeruginos*), gram-positve bacteria (*Micrococcus luteus*, *Mycobacterium phlei*, *Staphylococcus aureus*), yeasts (*Candida albicans*, *Hansenula anomala*, *Saccharomyces cerevisae*), and filamentous fungi (*Aspergillus fumigatus*, *Botrytis cinerea*, *Pythium debaryanum*). Agar diffusion assays were carried out using paper discs of 6 mm diameter spotted with 20 µg of the compound dissolved in MeOH. The compounds showed no activity against the test organisms. Stylissadines A (**5**) and B (**6**) showed also no cytotoxic activity (> 40 µg/mL) against L929 mice fibroblast cells (MTT assay).