Analysis of calcium-induced effects on the conformation of fengycin

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Abstract

A combination of CD, FT-IR, NMR and fluorescence spectroscopic techniques was applied to elucidate the conformation of fengycin, a natural lipopeptide with antifungal and eliciting activities, in a membrane-mimicking environment and to investigate the effect of calcium ions on the conformation.

We mainly observed that fengycin adopts a turn conformation and that the side chain of glutamate residues plays a key role on the stabilization of the peptide ring backbone conformation. More particularly, the binding of calcium ions by the carboxylic moieties has a consequence on the environment of the tyrosine residues. Our data suggest also an arrangement of fengycin molecules into "β-sheet like micelles" in a membrane-mimicking environment and the enhancement of this aggregating effect in presence of calcium ions.

The modulation of the fengycin conformation by the environmental conditions may influence its biological properties.

Keywords

Conformational analysis, calcium, cyclic lipopeptide, *Bacillus subtilis*, antimicrobial peptide.

Introduction

Fengycin is a natural lipopeptide produced by *Bacillus subtilis* strains. It is characterized by strong antifungal activities against filamentous fungi with a broad spectrum [1]. Its ability to stimulate systemic plant defense response has also been demonstrated [2]. The site of action of fengycin is supposed to be the plasma membrane of sensitive cells. Its hemolytic activity which does not restrict its application in the pharmaceutical field is significantly lower compared to those of many other agents [3-4]. This context stimulates researches on the production optimization of this lipopeptide [5-7] as well as on the understanding of the molecular mechanisms of its action for further potential uses. Fengycin is produced through a non-ribosomal pathway composed by multienzymatic complexes [8]. It is constituted by ten amino acids (eight of them forming a cyclic structure) and by a β-hydroxy fatty acid chain linked to the peptide cycle (Figure 1). The length and the ramification of acyl chain can be variable and two isoforms differing by the nature of the amino acid in position 6 of the peptide moiety have been identified [9].

Even though the primary structure of fengycin had been determined [1,9] and its physico-chemical properties have been partially investigated [10-15], no detailed conformational analyses have been performed. Since the conformational properties of biologically-active peptide compounds are supposed to play an essential role on their biological activities, this work aims to analyze the conformation of fengycin in membrane-mimicking environmental conditions and to investigate the role of calcium ions on the conformation of fengycin.

Results and Discussion

Conformational analyses of fengycin by CD and FT-IR spectra

Before investigating the influence of calcium ions on the secondary structure of fengycin, we characterized the latter in TFE (trifluoroethanol), a well-known membrane-mimicking solvent [16]. Two spectroscopic methods, CD and FT-IR were used. Figure 2 gives the CD spectrum of fengycin in TFE. As expected, this spectrum does not correspond to the conventional spectra of peptides which usually adopt α-helical or β-sheet conformation because of the cyclic structure of the lipopeptide hindering that kind of conformation. This spectrum shows a broad positive band with two peaks centered at 217 and 233 nm and a negative band centered at 198 nm. The presence of the positive band at 217 nm could be explained by the $n\pi^*$ transition occurred within D-amino acids [17]. Moreover, this band might also be due to an unconventional turn conformation adopted by the peptide cycle of fengycin [18]. Besides, the spectra of peptides composed of L-amino acids and adopting an inverse γ-turn conformation (γ_L^{inv}) show a broad negative band at ~230 nm [17-19].. The broad positive band at 233 nm could then be interpreted as the inverse γ-turn conformation involving D-amino acids. Another characteristic of the fengycin CD spectrum is the presence of a negative band at ~190 nm. This band reflects the $\pi\pi^*$ transition occurring within peptide bonds and is compatible with the presence of turn (γ or β) conformations. This band may also appear in the case of "β-sheet like micelles" defined previously [20]. In order to check the presence or absence of aggregated structures, we used FT-IR technique which is more sensitive to this kind of structures.

Figure 3 gives the 1700-1500 cm^{-1} region of the FT-IR spectrum of fengycin in TFE. The spectrum shows a wide band centered at 1662 cm^{-1} with a shoulder at 1644 cm^{-1} in the amide-I region. The deconvolution of amide-I band gives four bands at 1666 and 1641 cm^{-1} with high frequency contribution and at 1695 and 1617 cm^{-1} with low frequency contribution. In

the amide II region, a band composed of different frequency contributions at 1569, 1548 and $1517 cm^{-1}$ were also observed.

The main bands of amide-I region at 1666 and 1641 cm^{-1} are compatible with the presence of turn structures while the bands at 1695 and 1617 cm^{-1} could be assigned to the presence of aggregated structures such as “like β-sheet micelles”.

In the amide-II region, the band at 1569 cm^{-1} corresponds to the side chain of glutamate residues while the band at 1517 cm^{-1} is due to the side chain of tyrosine residues.

Fluorescence analyses of fengycin

Since fengycin has two tyrosine residues well-known to contribute to the intrinsic fluorescence of peptides, the fluorescence properties of fengycin have been examined in order to probe its conformational arrangement in a membrane-mimicking environment. Figure 4 shows the excitation and the emission spectra of the lipopeptide in TFE. When excited at 275 nm, the lipopeptide presents an emission band with a peak centered at 303 nm, corresponding to the emission spectra of tyrosine [21]. This confirms that the fluorescence of fengycin is mainly provided by the presence of tyrosine residues.

Quenching studies were carried out by recording the fluorescence spectra of fengycin in the presence of potassium iodide (KI) as quencher at different concentrations (data not shown). As expected, the intensity of the fluorescence decreased with increasing concentration of the quencher. To get more information on the accessibility of tyrosine residues, the Stern-Volmer constant (K_{SV}) value was calculated from the Stern-Volmer plot and was around 7.8 M^{-1} (Table 1).

Calcium-induced changes on the fengycin conformation by CD and FT-IR

Since fengycin has two charged glutamate residues which could be involved in divalent ion chelation as proposed for surfactin, another lipopeptide from *Bacillus subtilis* [20], we investigate, in a second step, the role played by calcium (Ca^{2+}) ions on the possible conformational changes of fengycin.

In figure 5, CD spectra of fengycin in TFE and in the presence of different Ca^{2+} concentrations are shown. The addition of calcium to the fengycin induces conformational changes depending on the ratio of calcium to fengycin ($R_{Ca/F}$). At $R_{Ca/F} < 1$, no significant changes were observed on the CD spectra. At $R_{Ca/F} = 1$, a shift of the negative band at 198 nm to 200 nm and the disappearance of the two positive peaks at 233 and 217 nm with the appearance of a peak at the 220 nm were observed. While $R_{Ca/F}$ was greater than 1, the negative band was shifted to 203 nm and its intensity was significantly decreased. Moreover, a positive band at 194 nm appeared and the intensity of the positive band at 220 nm was increased. It is expected that the two negatively charged glutamate residues bind calcium ions through electrostatic attractions. Significant changes on CD spectra arised when the $R_{Ca/F}$ reached 1. The weak positive band at 194 nm could be assigned to the formation of few sheet micelles as observed in the case of other lipopeptides [20]. It can be suggested that the addition of calcium to the fengycin could favor the aggregation of the lipopeptide as the electrostatic repulsions are reduced. When the $R_{Ca/F}$ increased, the shape of the band remains unchanged but intensity increased, suggesting a more aggregating-effect of calcium. Moreover, the strong positive band observed at 220 nm could be assigned to the presence of several structures such as β-sheet conformation and/or β-turns and/or γ-turns [22].

Figure 6 gives the FT-IR spectra of fengycin obtained at different $R_{Ca/F}$. The addition of calcium to fengycin in TFE gives rise to important changes in the amide-I region of the spectra. These changes are dependent on the $R_{Ca/F}$ confirming our findings by circular

dichroism. Up to $R_{Ca/F}$ = 1, no significant change was observed. At $R_{Ca/F}$ = 1, the shoulder observed at 1644 cm^{-1} increased and hence became a distinct peak. So, we obtained a wide amide-I band with two equivalent peaks at 1662 and 1644 cm^{-1}. These peaks are compatible with turn conformation for the lipopeptide. The existence of two peaks could be attributed to the presence of two populations of peptide carbonyl groups. The first population at 1662 cm^{-1} represents the carbonyl groups which are less involved in hydrogen bonds while the peak at 1644 cm^{-1}, the carbonyl groups more involved in hydrogen bonds [20, 23]. The latter increased at $R_{Ca/F}$ = 1. Since the involvement of carbonyl groups in hydrogen bonds is directly related to the conformation adopted by the peptide part of fengycin, the calcium has thus an influence on the fengycin conformation. When $R_{Ca/F}$ reached 2, a band centered at 1646 cm^{-1} with a shoulder at 1661 cm^{-1} was observed. The increase of $R_{Ca/F}$ seems to favor the increase of the hydrogen-bounded peptide carbonyl group population. The major band at 1646 cm^{-1} can be therefore associated with β-turns while the band at 1661 cm^{-1} can be associated with γ-turns [20]. When $R_{Ca/F}$ reached 5, the amide-I band shifted to 1635 cm^{-1}. This band could be attributed to C_7 bend structures which are supposed to be equivalent to γ-turns or to the amide groups complexed to Ca^{2+} [17, 20]. Despite the important changes occurring in the amide-I region, the amide-II region of the spectra seems to be not significantly affected by the addition of calcium. However, a shift of the tyrosine band was observed when $R_{Ca/F}$ increased. Compared to the pure fengycin spectrum, a slight shift occurred when $R_{Ca/F}$ ~1 but it became more important when $R_{Ca/F}$ reached 5.

Influence of the calcium on the fengycin fluorescence

In order to understand whether calcium-induced conformational changes have a consequence on the environment of the tyrosine residues, the fluorescence of the calcium/fengycin complex was measured at $R_{Ca/F}$ =1 (data not shown). A slight shift of the maximum emission from 303

nm to 305 nm, corresponding to the modification of the environment of the tyrosine residues, was observed in the presence of calcium. To get more information on the accessibility of the tyrosine residues, quenching experiments have been conducted in the presence of calcium.

Table 1 compares the K_{SV} values of pure fengycin, fengycin in the presence of calcium at $R_{Ca/F}$ =1 and at $R_{Ca/F}$ =5 obtained from the Stern-Volmer plots for each sample. In the presence of calcium, the K_{SV} value was slightly higher suggesting an increased accessibility of tyrosine residues. The lipopeptide seems then to adopt a conformation leading to tyrosine residues to be more accessible in the presence of calcium.

Influence of calcium ions on the fengycin NMR spectra

In order to evaluate the influence of calcium ions on the conformation of fengycin, combined application of various 1D and 2D NMR experiments including ^{1}H–^{1}H DQCOSY, HSQC, HMBC and TOCSY analyses were performed in deuterated TFE.

Careful inspection of 2D NMR ^{1}H-^{1}H COSY and TOCSY spectra enabled to sequence all the proton signals in different spin systems belonging to the ten residues, namely glutamic acid (Glu, 2x), ornitine (Orn), threonine (Thr), alanine (Ala), proline (Pro), glutamine (Gln), tyrosine (Tyr, 2x) and isoleucine (Ile). Furthermore, the presence of a methine signal at 4.09 ppm and of a methyl group at 0.87 ppm in the 600 MHz ^{1}H-NMR spectrum confirmed the presence of the β-hydroxyl fatty acid side chain. Association of all proton signals with those of the directly linked carbons was obtained by analysis of the 2D HSQC spectrum. Finally, study of the correlation peaks from the 2D HMBC spectrum allowed to connect the spin systems, confirming therefore the lipopeptide structure of fengycin (Fig. 1).

These analyses allowed us a total assignment of the proton and carbon NMR signals of fengycin and were in agreement with the values reported in previous literature for this

compound [9]. With these results in hand, NMR analyses in the presence of calcium ions could be achieved in the same experimental conditions.

Once again, the ten amino acids and the β-hydroxy fatty acid side chain were identified on the basis of 2D NMR DQCOSY, TOCSY, HSQC and HMBC analyses.

Interestingly, marked differences could be observed between the spectra recorded with and without calcium.

Comparison of the 2D COSY spectra showed indeed a clear shift of the Tyr-3 aromatic protons, from, respectively, δ 6.80 and 7.05 to δ 7.10 and 7.25 in the presence of calcium ions (Figure 7). Moreover, several other amino acid protons were affected by the presence of calcium ions: the Ala methine and methyl groups [from δ 1.38 (CH_3) and 4.47 (CH) to δ 1.34 and 4.75, respectively], the Thr methyl group [from δ 1.17 to δ 0.97], a Pro methylene [from δ 3.67 to δ 4.35] and the non aromatic protons of Tyr-9 [from δ 3.10 (CH_2) and 4.51 (CH) to δ 3.02 and 4.76, respectively] (Figure 7).

Although less obvious, analysis of the 2D HMBC spectra showed also the influence of calcium ions on the chemical shifts of several amino acid carbons. When compared with the NMR data of fengycin, the chemical shifts of the Tyr-1 aromatic carbons, the Ile and Glu carbonyl groups, and the Thr and Ile methine signals are affected by the presence of calcium ions (Figure 8).

Those results clearly indicate that calcium ions have an influence on the protons and carbon chemical shifts of several amino acids. This could be explained by a conformational change of fengycin. The chelation of anionic carboxylates by the divalent calcium cation leads to the formation of a bridge between the glutamate and force some residues, namely *allo*-Thr, Ala, Pro and both Tyr, in the cyclic substructure to come closer to each other (Figure 9), affecting therefore their protons and carbons chemical shifts.

This hypothesis was reinforced by NOESY analyses of fengycin in the presence of calcium ions. Most noteworthy were the correlation observed between the Ala methyl and methine groups (δ 1.34 and 4.75, respectively) and the Thr CHOH (δ 3.59), between an aromatic proton belonging to Tyr-3 (δ 7.10) and the Thr methyl group (δ 0.97), and between the aromatic Tyr-3 proton at δ 7.25 and a Pro methylene (δ 4.35) (Figure 9 and supplementary data), correlation which were not observed in the spectra recorded without calcium.

Those NMR results confirm the binding of the calcium ions by the two charged glutamates and are also in agreement with the CD, FT-IR and fluorescence analyses, showing that the presence of calcium ions has an effect on the environment of the tyrosine residues.

Concluding Remarks

Fengycin is a natural and biologically-active molecule. As it is well known that the conformation of molecules plays a crucial role in their activity, the aim of this work was first to analyse the conformational properties of fengycin. Secondly, the effect of calcium ions on these properties was evaluated since divalent ions are often present in biological media.

Our findings indicate that fengycin was able to adopt different conformations depending on the environmental conditions. All spectroscopic data put emphasis on the role of the glutamate side chain groups on the stabilization of the peptide ring backbone conformation. The presence of this kind of chelator groups, the ability of the lipopeptide to form micelles and an environment-adapted peptide backbone conformation must play an important role for the biological activity of fengycin as antifungal, hemolytic or elicitor. For example, the membrane-destabilizing effect of fengycin giving rise to its biocide or elicitor activity could be favored in the presence of divalent ions by adopting a more appropriate conformation. Moreover, the withdrawal of calcium ions which are important for many biological processes

could also disturb the cell environment and influence the biocide or elicitor activity. Future work will be carried out on the effect of calcium on the fengycin-membrane interactions.

Experimental section

Materials

Calcium perchlorate (tetrahydrate), trifluoroethanol (TFE) (NMR grade), potassium iodide (KI) and sodium sulfate (Na_2SO_3) were provided from Sigma Aldrich. The purity of all solvents was at least 99% and the solvents were used without further purification. The ultrapure water was provided from Millipore system (Bedford, MA) and had a resistivity of 18.2MΩ. Fengycin was produced by fermentation of the *Bacillus subtilis* strain S499 in an optimized culture media as described by Jacques et al. [24] and extracted in a semi-preparative scale from the culture medium by solid-phase extraction on Bond Elut C18 (50 g, Varian CA), as previously described [25]. The crude extract was applied to a silica gel 60 column (30× 2.5 cm, 45 g, 250–325 mesh, Merck, Darmstadt, Germany) for separating fengycin from surfactin and iturin A by flash chromatography. Fengycin was eluted with chloroform/methanol/water/ethanol (7/3/1.5/3.5, by volume) after elution of surfactin and iturin A. The identification and verification of the purity were made by amino acid analysis, analytical RP-HPLC, and MALDI-TOF mass spectrometry (Ultraflex TOF, Bruckner, Karlsruhe, Germany). Fengycin is composed of one isoform compound (isoform A with D-Ala, Fig. 1), containing homologous molecules comprising between 14 and 18 carbon atoms. The HPLC-UV (214 and 280 nm) purity of the compound was higher than 95%.

Infrared Spectroscopy (FT-IR)

Infrared spectra were recorded by means of a Bruker Equinox 55 spectrometer (Karlsruhe, Germany) equipped with a liquid nitrogen-cooled Mercury-Cadmium-Telluride detector after

128 scans at 4 cm^{-1} resolution. During the data acquisition, the spectrometer was continuously purged with filtered dried air. All the experiments were performed with a demountable cell (Bruker) equipped with CaF_2 windows [23]. The solvent spectrum was subtracted from the sample spectrum taken under the same conditions. The solvent contribution in the amide-I region due to the water traces in TFE corresponding to the band at 1633 cm^{-1} was removed by referring on the other absorption band at 3688 cm^{-1} as described in [20]. The concentration of the fengycin in TFE was 20 mg/mL. Each spectrum is the representative of at least three independent measurements. For curve fitting analyses, the spectra of pure fengycin in TFE was fitted by the means of Lorentzian band shape as described in [26-27].

Circular dichroism (CD)

Far-UV CD spectra of fengycin samples between 190-260 nm were collected using a Jasco Circular Dichroism Spectrometer model J-810 (Easton, MD) at room temperature (20°C ±1) with a 1 mm optical path length quartz cell. The lipopeptide concentration was fixed at 200 µM in TFE. All CD experiments were repeated at least two times to ensure the reproducibility. The solvent contribution was removed by subtracting the spectrum of TFE taken under the same conditions. Each spectrum is the representative of at least three independent measurements and was smoothed by the means of means-movement algorithms available with Jasco software.

Fluorescence spectroscopy

Fengycin fluorescence was measured using a LS-50 B Perkin Elmer fluorimeter (Waltham, MA). The excitation wavelength was fixed at 275 nm which gives a maximal emission at 303 nm. The concentration of fengycin was fixed at 3.5 µM. Quenching studies were performed by using KI as quencher. KI solution was prepared in water at 2.5 M in order to minimize the

volume (< % 0.6 of total volume) of KI solution adding in the solution of fengycin in TFE. Na_2SO_3 at about 50 µM was added to avoid the oxidation of iodide. Quenching parameters were calculated from the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of the quencher respectively, $[Q]$ is the concentration of quencher and K_{SV} is the Stern-Volmer constant. The slope of the Stern-Volmer plot gives the K_{SV} value giving the information on the accessibility of the fluorophore.

Nuclear Magnetic Resonance Spectroscopy

The 1D and 2D NMR analyses of fengycin were carried out with a Varian 600 MHz spectrometer equipped with a 5-mm high resolution AutoX Indirect detection probe. The spectra were obtained from 21.5 mg of purified biosurfactant dissolved in 500 µL deuterated TFE (Euriso-Top) at 298 K in a 5 mm ultra precision NMR sample tube equipped with a coaxial insert for $CDCl_3$ external lock (Euriso-Top). For the NMR analyses in the presence of calcium, 4.2 mg of calcium perchlorate were added to the sample (1:1 molar ratio). Chemical shifts are given on the δ-scale relative to sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate (TSP) (Sigma Aldrich).

For ^1H NMR at 600 MHz, 1024 scans were acquired with a 2 s relaxation delay. The 90 ° pulse was 6.5 µs at 30 dB. For ^{13}C NMR at 150 MHz, 10000 scans were acquired with a 2 s relaxation delay and the 90 ° pulse was 13.6 µs at 60 dB. ^1H–^1H 2D gDQCOSY spectrum was acquired using a standard Varian pulse sequence (16 scans) with a relaxation delay of 2.0 s and a spectral width in both dimensions of 9615.4 Hz. The 2D gHSQCAD and 2D

gHMBCAD spectra were recorded using standard Varian pulse programs with $^{1}J_{(C,H)}$ = 146 Hz, f_2 9615.4 Hz and f_1 30165.9 Hz, and a relaxation delay of 2.5 s (32 scans) ; $^{n}J_{(C,H)}$ = 8 Hz, f_2 9615.4 Hz and f_1 36199.1 Hz, and a relaxation delay of 2.0 s (64 scans), respectively. The z-TOCSY experiment was obtained using a spin lock mixing time of 80 ms and a relaxation delay of 2.0 s (32 scans). The NOESY spectra were acquired using mixing times of 120 and 250 ms and a relaxation delay of 2.0 s [28].

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Table

Table 1. *Ksv* values for the quenching of pure fengycin , at $R_{Ca/F}$=1 or at $R_{Ca/F}$=5.

	***Ksv* value (M^{-1})**
Pure fengycin	7.8 ± 0.5
$R_{Ca/F}$ =1	9.5 ± 1.1
$R_{Ca/F}$ =5	14.2 ± 2.0

Caption Legend

Figure 1. Primary structure of fengycin.

Figure 2. CD spectrum of fengycin in TFE.

Figure 3. Curve Fitted FT-IR spectrum of fengycin in TFE.

Figure 4. Excitation (left) and emission (right) spectra of fengycin in TFE

Figure 5. CD spectra of fengycin in TFE at different ratios of calcium to lipopeptide: (——) Pure fengycin, (— —) $R_{Ca/F} = 0.2$, (– –) $R_{Ca/F} = 0.4$, (······) $R_{Ca/F} = 1$,(— – —) $R_{Ca/F} = 2$, (– · –) $R_{Ca/F} = 5$.

Figure 6. FT-IR spectra of fengycin in TFE at different ratios of calcium to lipopeptide: (——) Pure fengycin, (— —) $R_{Ca/F} = 0.2$, (······) $R_{Ca/F} = 1$,(– –) $R_{Ca/F} = 2$,(– · –) $R_{Ca/F} = 5$.

Figure 7. Comparison of the fengycin 2D NMR COSY spectra recorded without calcium perchlorate (blue) and with calcium perchlorate (red).

Figure 8. Comparison of the fengycin 2D NMR HMBC spectra recorded without calcium perchlorate (blue) and with calcium perchlorate (red)

Figure 9. Specific NOESY correlation observed in the NMR spectrum of fengycin in the presence of calcium ions

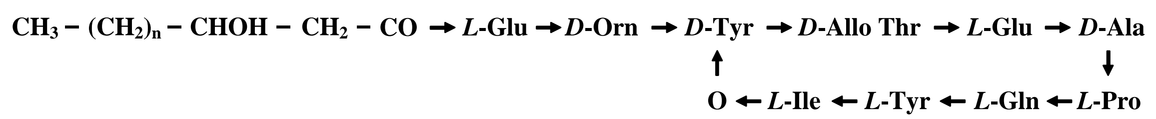


Figure 1

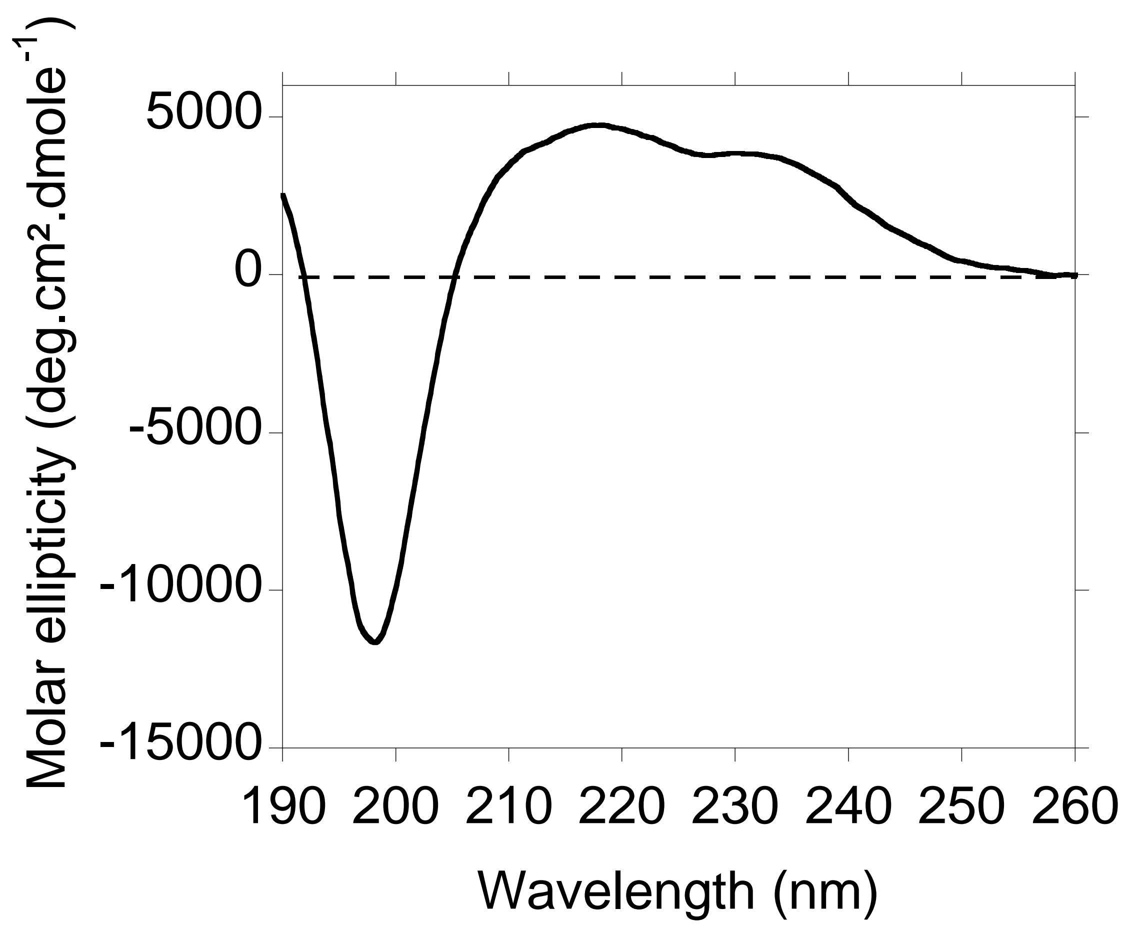
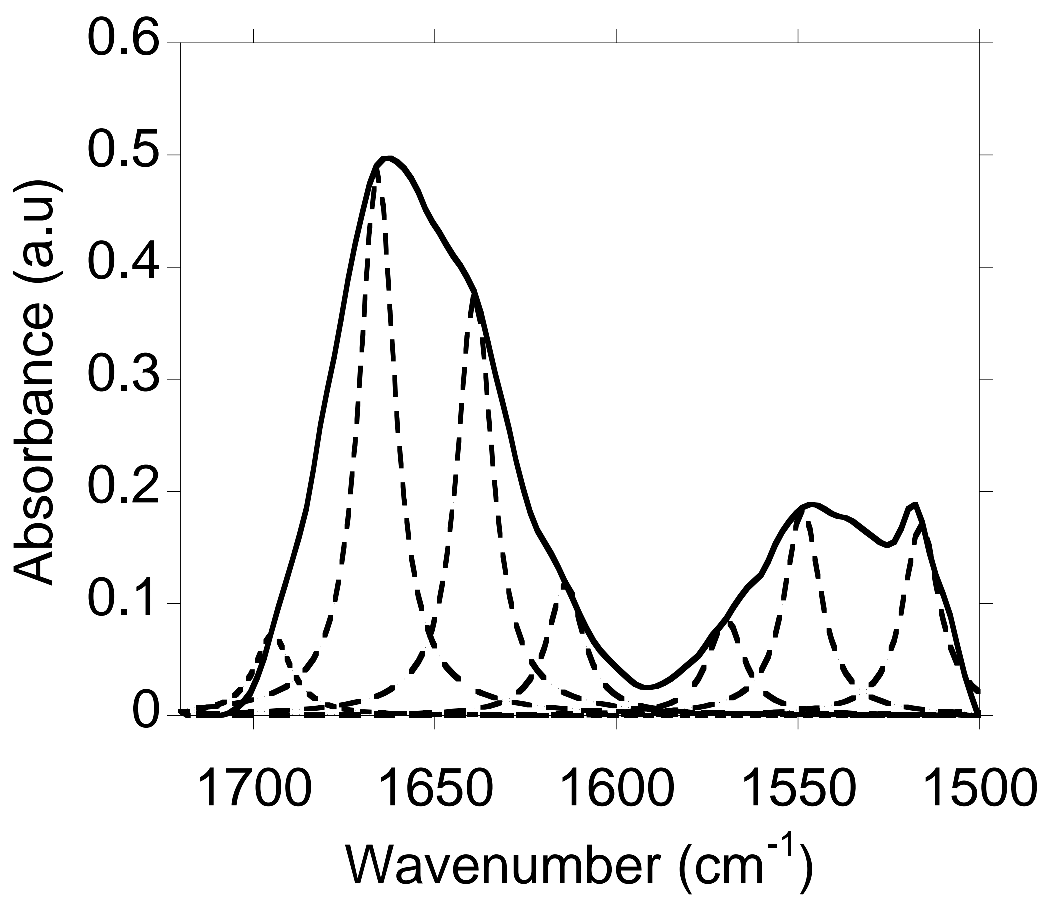


Figure 2



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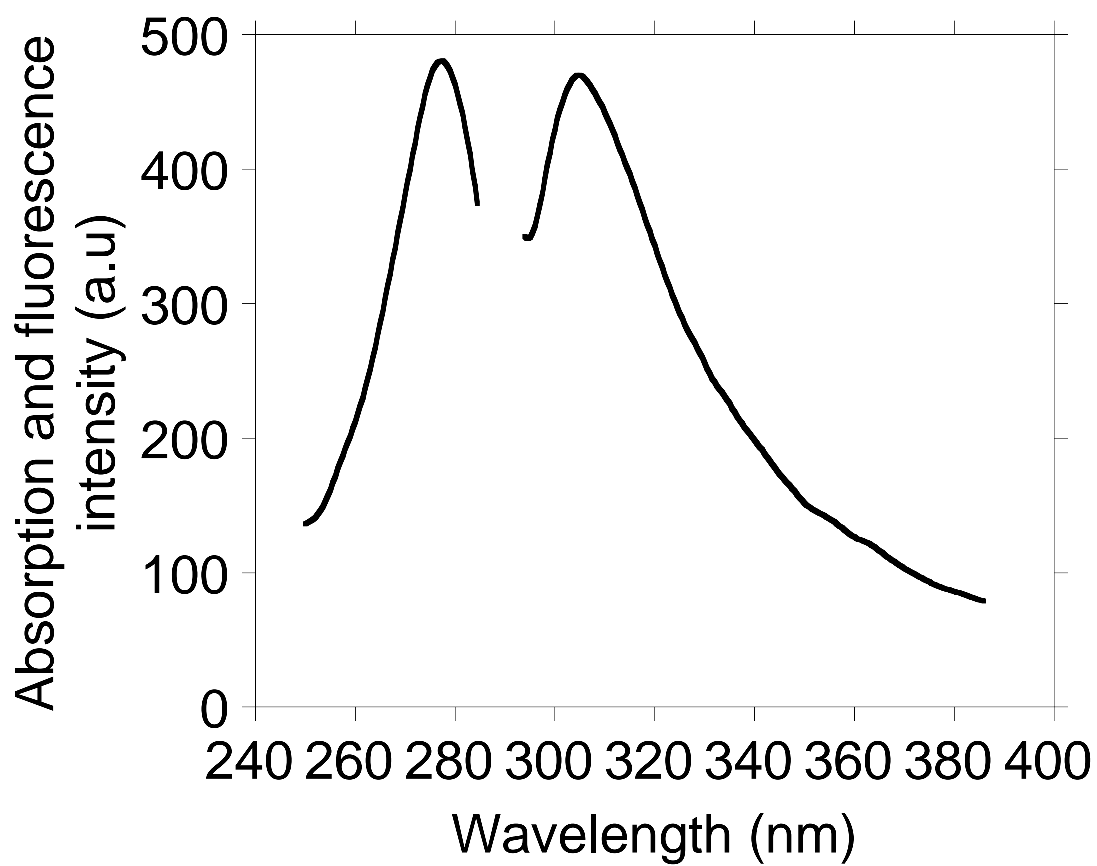

Figure 4

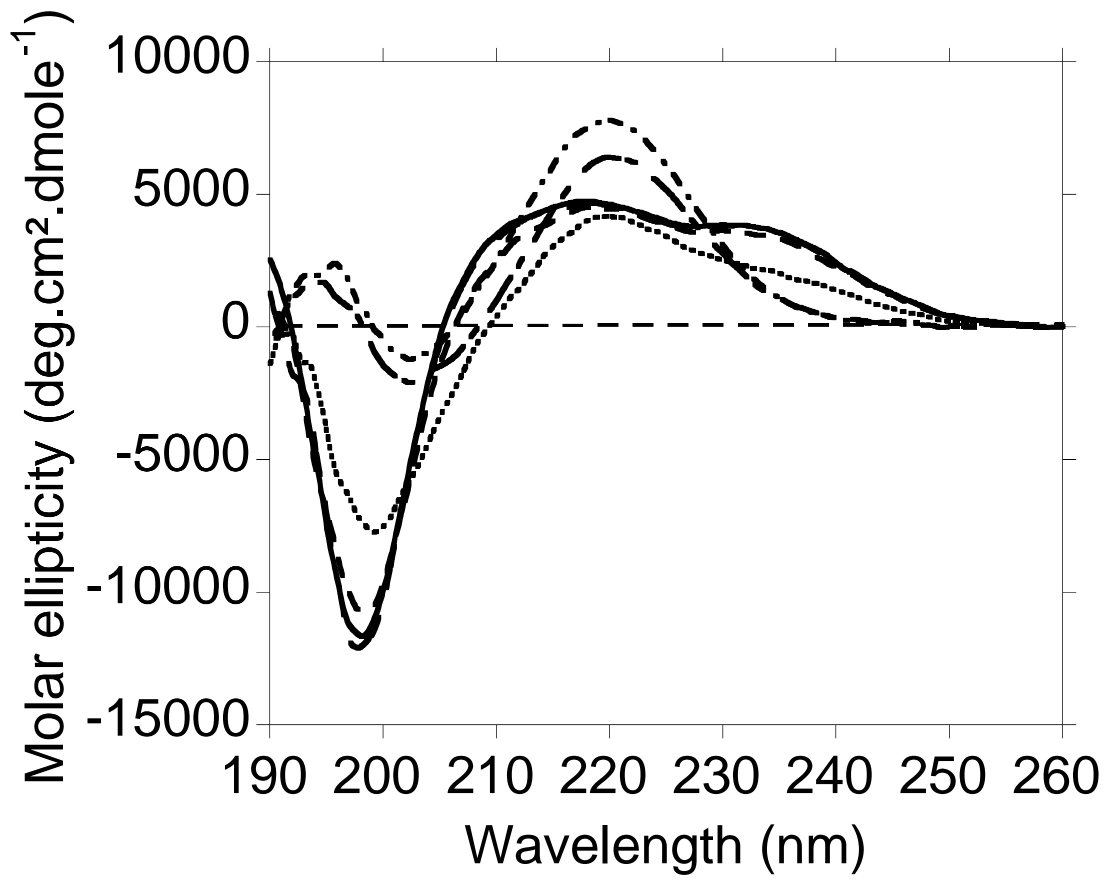

Figure 5

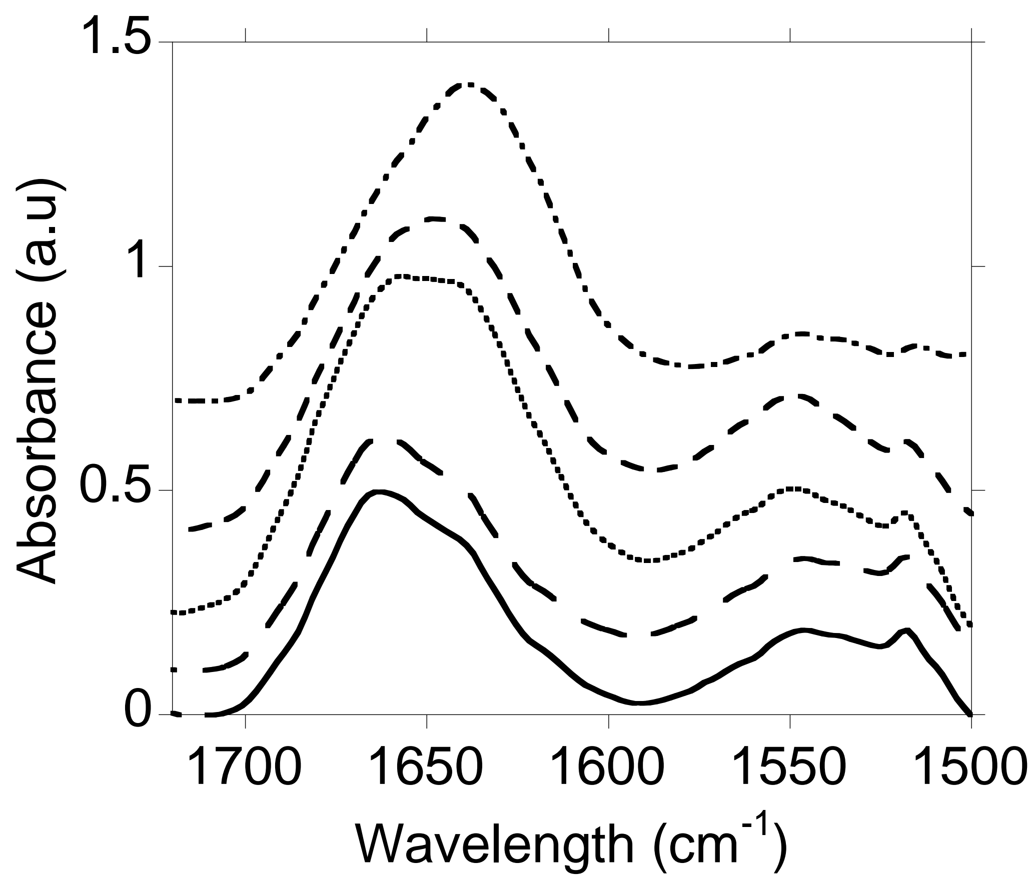

Figure 6

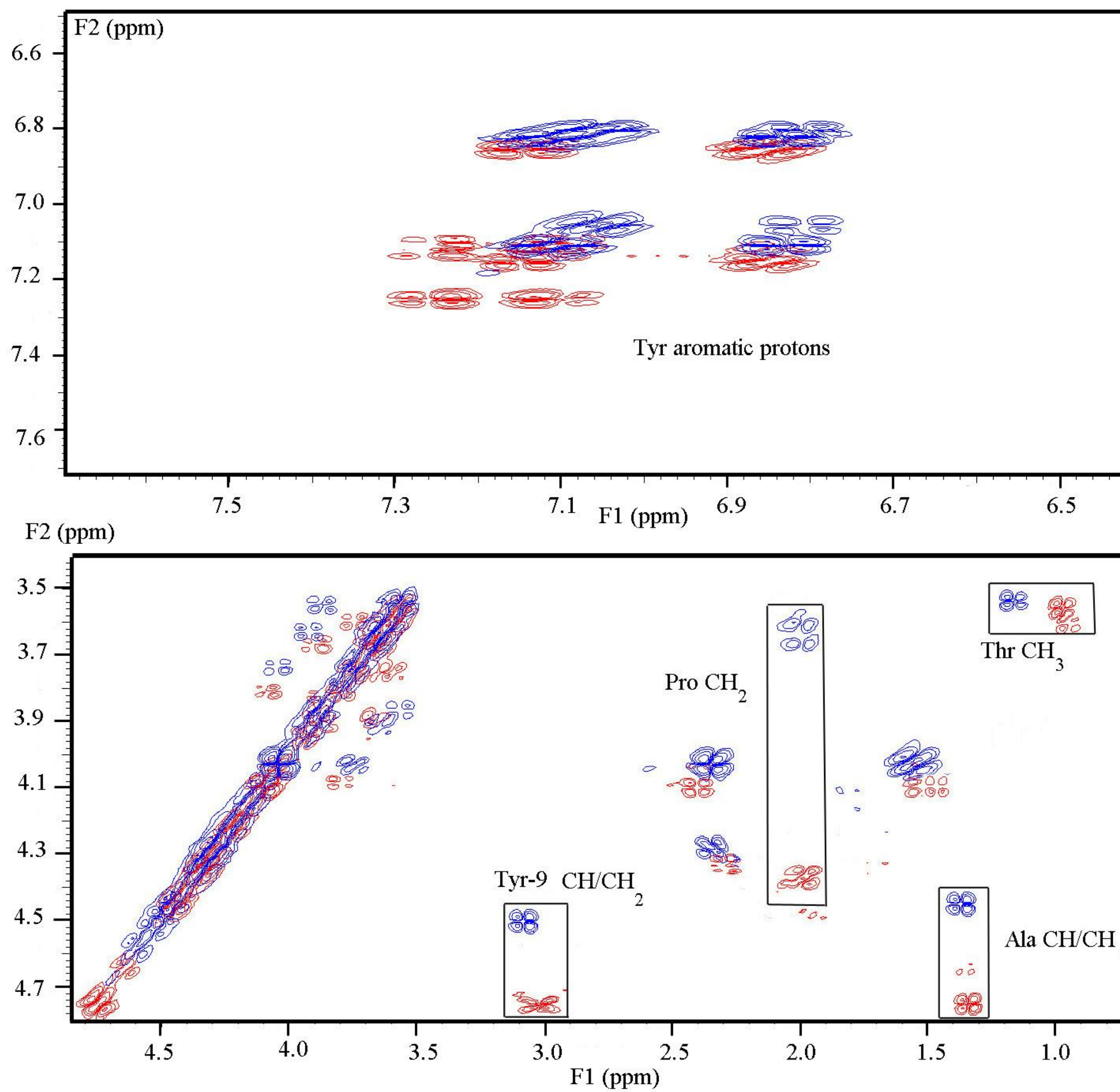

Figure 7

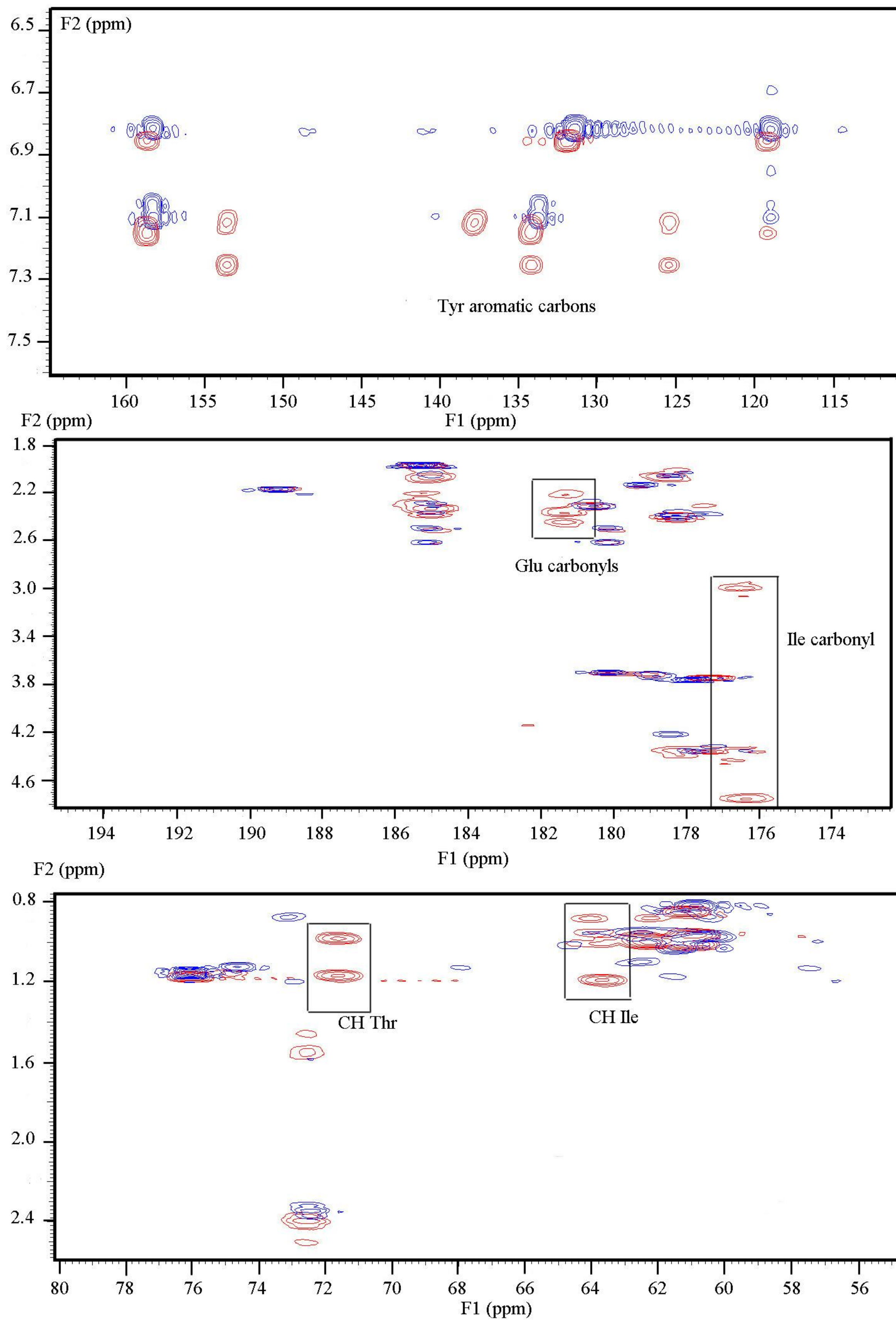

Figure 8

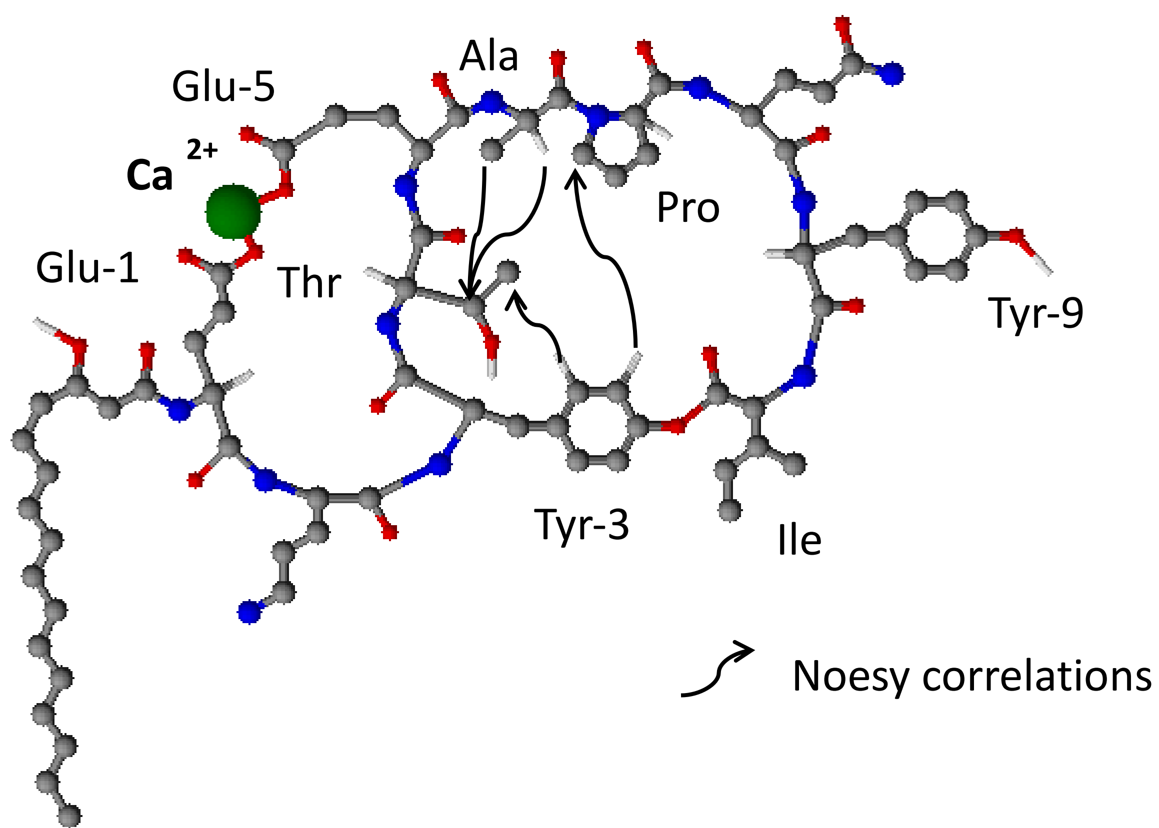

Figure 9

Supplementary data

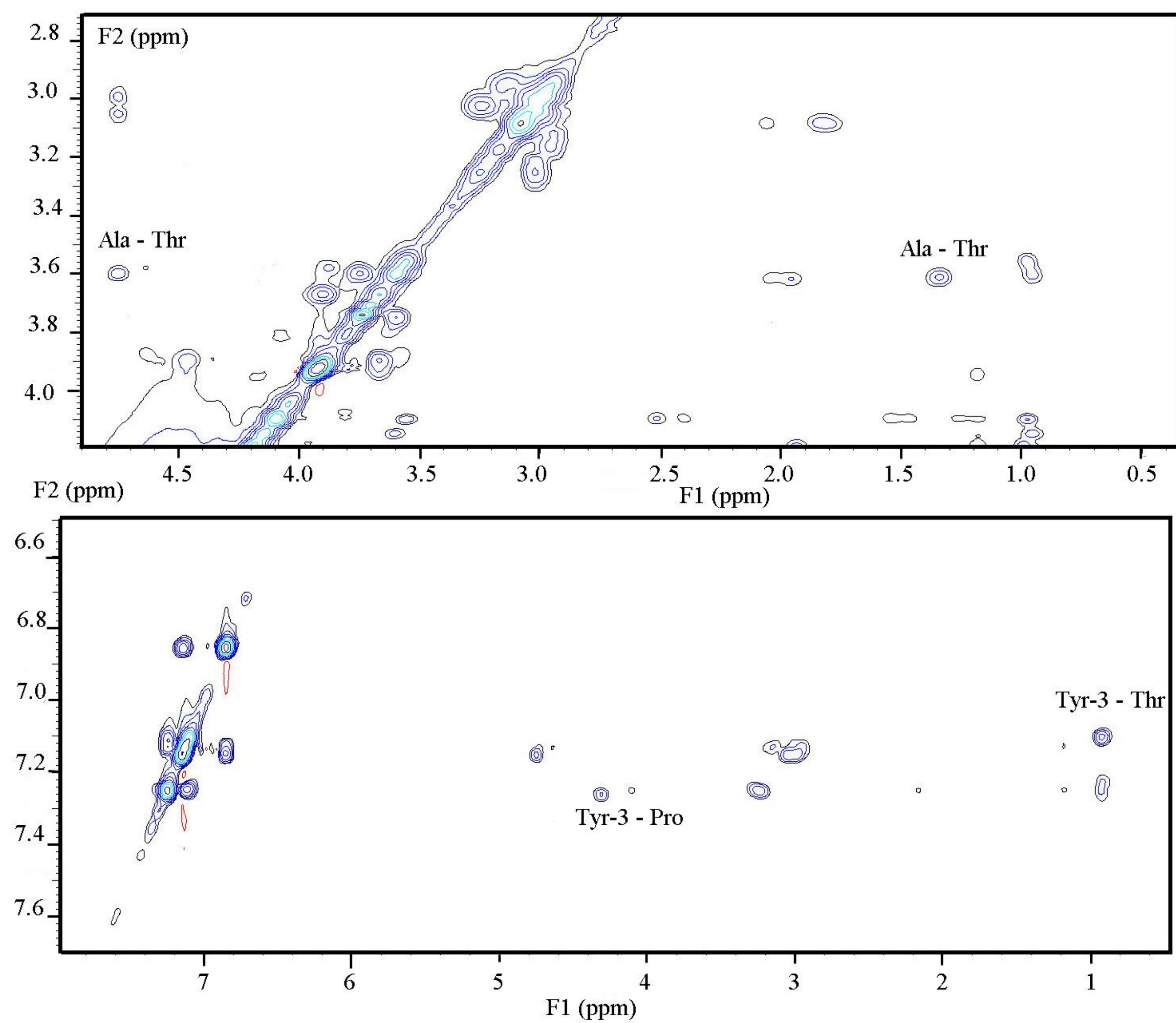

Figure. NOESY spectrum of fengycin recorded in the presence of calcium perchlorate

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Fengycin conformation

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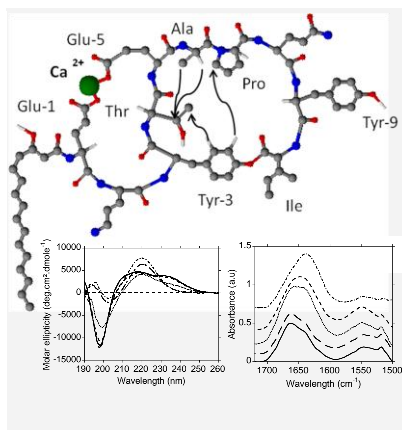
Analysis of calcium-induced effects on the conformation of fengycin



Representation of fengycin conformation in presence of calcium ion showing the specific NOESY correlation observed by NMR and in accordance with CD, FT-IR and fluorescence spectroscopic data

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