Electronic Supplementary Material (ESI) for ChemComm.

Boosting the sensitivity of in vitro β-lactam allergy diagnostic tests

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EXPERIMENTAL

1 General Experimental

NMR Spectra: ^{1}H and ^{13}C-NMR spectra were recorded on Bruker AVIIIHD NanoBay 400MHz spectrometer using TOPSPIN software. Proton and carbon chemical shifts ($\delta^{1}H$, $\delta^{13}C$) are quoted in ppm and referenced to tetramethylsilane with residual protonated solvent as the internal standard. Resonances are described using the following abbreviations; s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet), br (broad), app (apparent), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz.

Mass Spectra: High resolution mass spectra were recorded using a AB SCIEX TripleTOF™ 5600 LC/MS/MS System. The LC system used was an Agilent 1290 HPLC system. The analyses were performed using an Agilent EC-C_{18}. The date acquisition used are in positive mode, over a mass range of 100 – 950 m/z. The MS was using an IDA acquisition method with: the survey scan type (TOF-MS) and the dependent scan type (product ion) using 35V of collision energy. Data was evaluated using the qualitatively evaluated using the Peak View™ software. m/z values are reported in Daltons; high resolution values are calculated to four decimal places from the molecular formula, all found values being within a tolerance of 5 ppm.

Chromatography techniques: TLC was performed on Merck Glass TLC silica gel 60 F_{254} 0.2 mm precoated plates and visualised using ultraviolet light and potassium permanganate stain.

MS-MALDI-TOF: The samples were analyzed in a 5800 MALDI TOF-TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 15000-100000 m/z.

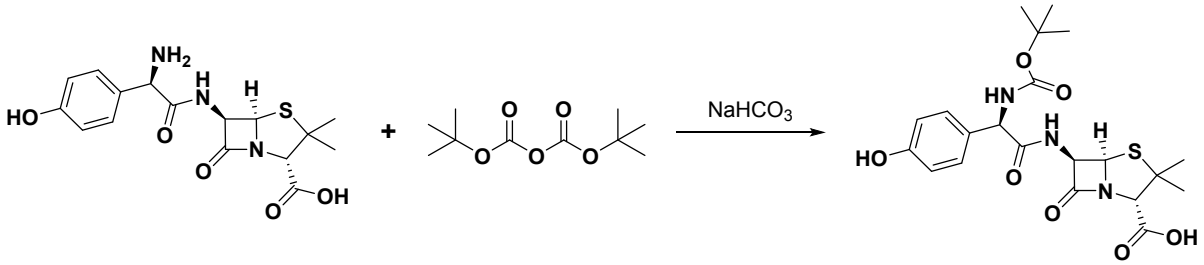
2. Chemicals, immunoreagents and buffers

Benzylpenicillin sodium salt, amoxicillin trihydrate, aztreonam (AZT), 1,3-diaminopropane, 1,5-diaminopentane, 1,7-diaminoheptane, ethylene diamine dihydrochloride, 1,4-diaminobutane dihydrochloride, 1,4-phenylenediamine dihydrochloride, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), anhydrous di-tert-butyl dicarbonate ($(Boc)_2O$), sodium bicarbonate, Tween 20, human serum albumin (HSA), histone from calf thymus (H1) and keyhole limpet hemocyanin (KLH) are from Sigma-Aldrich (Madrid, Spain). Dichloromethane (DCM), N,N-dimethylformamide (DMF), dioxane, ethyl acetate, hydrochloric acid 37 % (HCl) and buffer salts are from Scharlau (Sentmenat, Spain) and used without further purification. Deuterated dimethyl sulfoxide (DMSO-d_6) is from ACROS Organics (New Jersey, USA). Specific anti- β-lactam IgE artificial human sera (ARTHUS) to PG is kindly provided by Dr. Fooke (Neuss, Germany) and anti-human IgE monoclonal antibody is from Ingenasa, S.A. (Madrid, Spain). Polyclonal secondary antibody goat α-rabbit IgG labelled with horseradish peroxidase (GAR-HRP) and goat anti-mouse antibody labelled with horseradish peroxidase (GAM-HRP) are purchased from Abcam (San Francisco, USA). IgE human serum (3rd WHO International Standard) is purchased from National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK) and tetramethylbenzidine (TMB) substrate is from SDT GmbH (Baesweiler, Germany). Omalizumab antibody and Coomassie Brilliant Blue R-250 staining solution are from Bio-Rad (Madrid, Spain). Amicon Ultra 0.5 pre-concentred 10 K filters are from Fisher Scientific (Madrid, Spain).

The employed buffers are: (I) sodium phosphate buffer 0.1 M, sodium chloride 0.15 M, pH 7.2; (II) MES 0.1 M, pH 4.7; (III) sodium carbonate 0.5 M, pH 11.0; (IV) phosphate buffer saline (PBS 1X, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.4); (V) PBS-T (PBS 1X containing 0.05% Tween 20); and (VI) sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, as printing buffer. All buffers are filtered through a 0.45-µm pore size nitrocellulose membrane from Thermo Fisher Scientific (Madrid, Spain) before being used.

3. Synthesis of BLC-derived haptens

Boc-amoxicillin



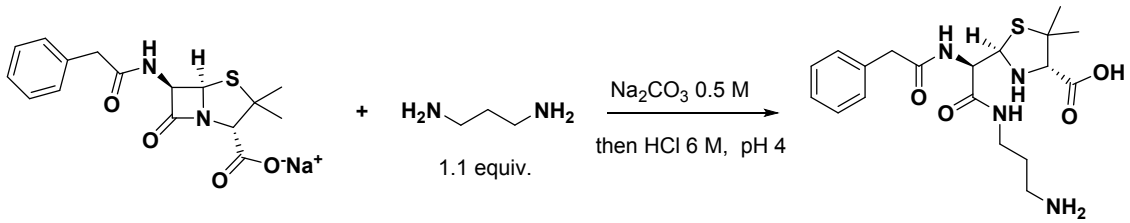
$NaHCO_3$ (200 mg, 2.38 mmol, 2 equiv.) is added to a solution of amoxicillin (440 mg, 1.19 mmol) in 150 mL H_2O and stirred at 5 ºC. Anhidrious $(Boc)_2O$ (390 mg, 1.79 mmol, 1.5 equiv.) dissolved in 1.5 mL dioxane is added dropwise and the mixture is allowed to react at 0 ºC for 1 h and then overnight at room temperature. The organic layer is extracted and washed twice with EtOAc and saturated $NaHCO_3$, respectively. Aqueous layers are mixed and acidified with HCl 6 M to pH 1 and extracted three times with EtOAc. Organic layers are collected and dried over $NaSO_4$. The solvent was evaporated and the desired compound dried under reduced pressure. Yield: 97%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.36 (s, 1H), 8.73 (d,

J = 7.9 Hz, 1H), 7.18 (d, J = 9.7 Hz, 2H), 6.66 (d, J = 8.3 Hz, 2H), 5.45 (dd, J = 7.8, 4.2 Hz, 1H), 5.37 (d, J = 4.2 Hz, 1H), 5.25 (d, J = 8.5 Hz, 1H), 4.15 (s, 1H), 1.91 (s, 1H), 1.55 (s, 3H), 1.41 (s, 3H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.30, 170.46, 168.99, 156.80, 128.46, 114.86, 78.16, 70.47, 67.50, 66.36, 64.19, 59.80, 57.91, 30.52, 28.19, 26.71, 13.85. HRMS (ESI-TOF) m/z calculated for $C_{21}H_{27}N_3O_7S$ ([M+H$^+$]): 466.1642, found 466.2539.

3.1. BLC-oyl derivates

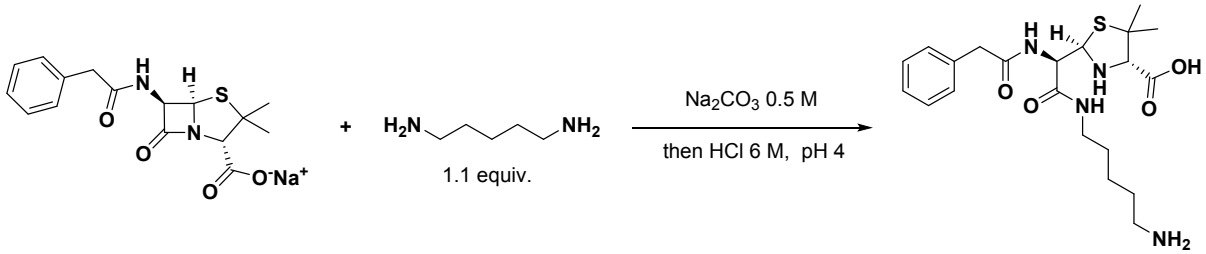
0.150 mmol of the corresponding BLC dissolved in deionized water (0.1 M) was basified by slow addition of sodium carbonate 0.5 M, pH 11.0. Then, 0.165 mmol (1.1 equiv) of the corresponding diamine was added dropwise at 0 ºC under nitrogen atmosphere. The reaction was stirred at room temperature for 3 h followed by an acidification to pH 4.0 with HCl 6.0 M. The solution was filtrated and the desired precipitate washed twice with acidified water. The compound was allowed to dry under vacuum to give a white powder that was characterized by NMR and MS and used without further purification. Yield of the reaction was corrected by measuring the amount of salts (Na_2CO_3 and NH_4Cl) present. Haptens **1-6** (Scheme S1) were obtained though this route.

*Hapten **1***



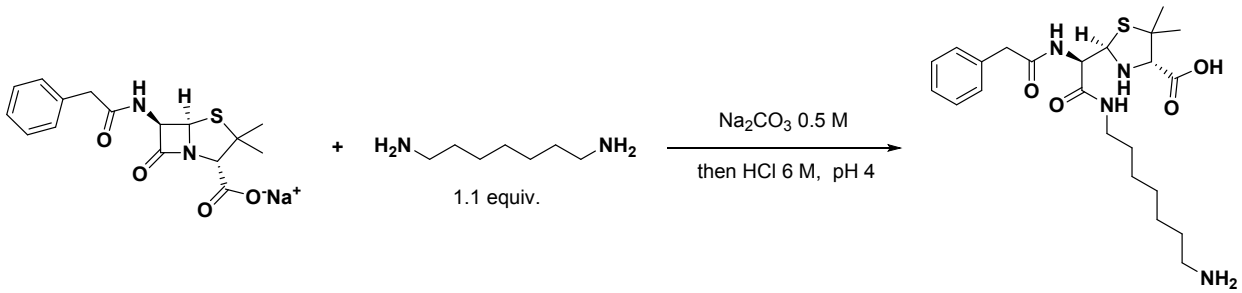
Yield: 97%. HRMS (ESI-TOF) m/z calculated for $C_{19}H_{28}N_4O_4S$ ([M+H$^+$]): 409.1904, found 409.1906.

*Hapten **2***



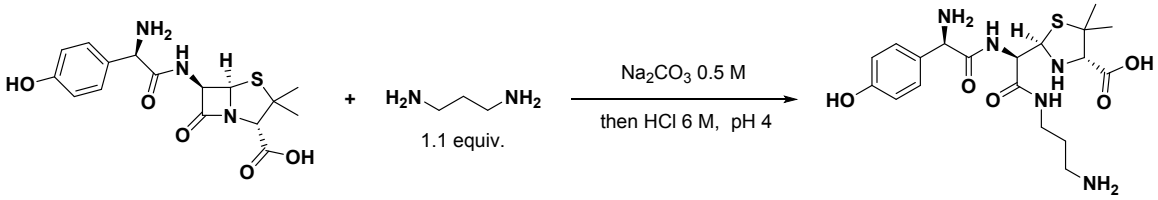
Yield: 61%. HRMS (ESI-TOF) m/z calculated for $C_{21}H_{32}N_4O_4S$ ([M+H$^+$]): 437.2217, found 437.2222.

*Hapten **3***



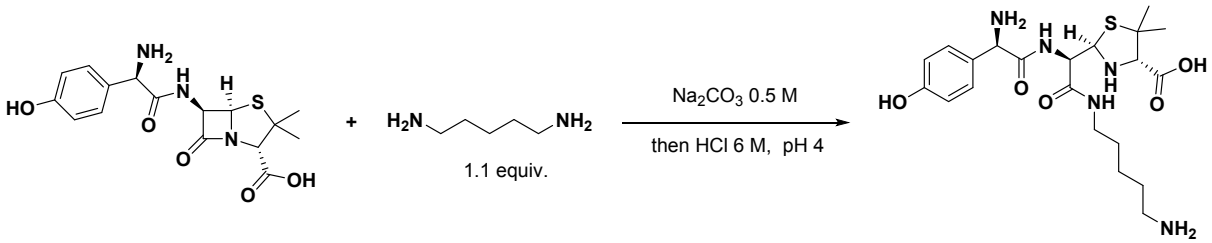
Yield: 77%. HRMS (ESI-TOF) m/z calculated for $C_{23}H_{36}N_4O_4S$ ([M+H$^+$]): 465.2530, found 465.2527.

*Hapten **4***



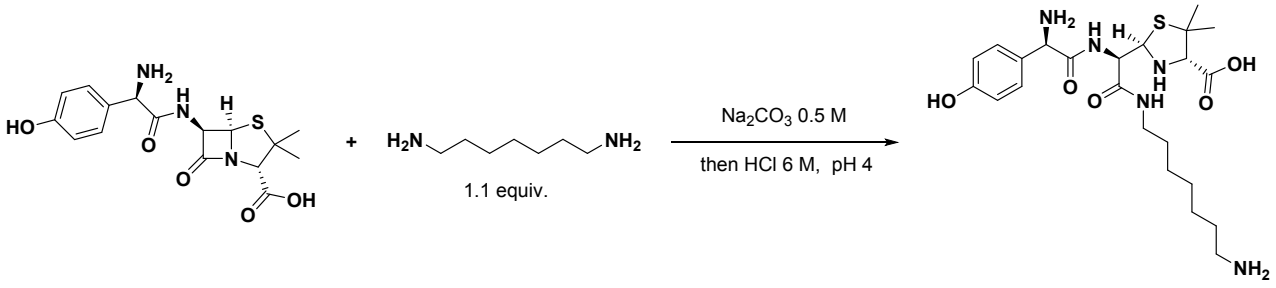
Yield: 100%. HRMS (ESI-TOF) m/z calculated for $C_{19}H_{29}N_5O_5S$ ([M+H$^+$]): 440.1962, found 440.1971.

*Hapten **5***



Yield: 80%. HRMS (ESI-TOF) m/z calculated for $C_{21}H_{33}N_5O_5S$ ([M+H$^+$]): 468.2275, found 468.3913.

*Hapten **6***

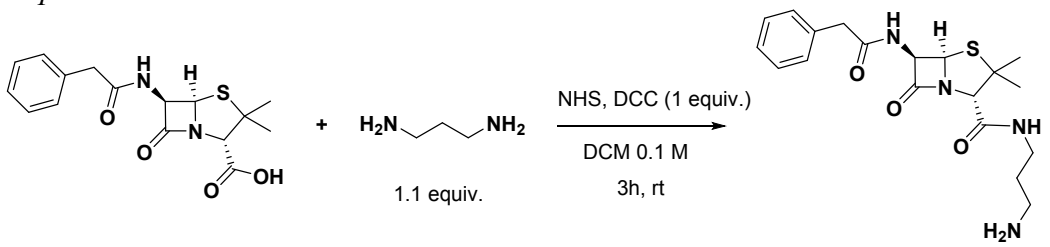


Yield: 100%. HRMS (ESI-TOF) m/z calculated for $C_{23}H_{37}N_5O_5S$([M+H$^+$]): 496.2588, found 496.2589.

3.2. BLC-anyl derivates

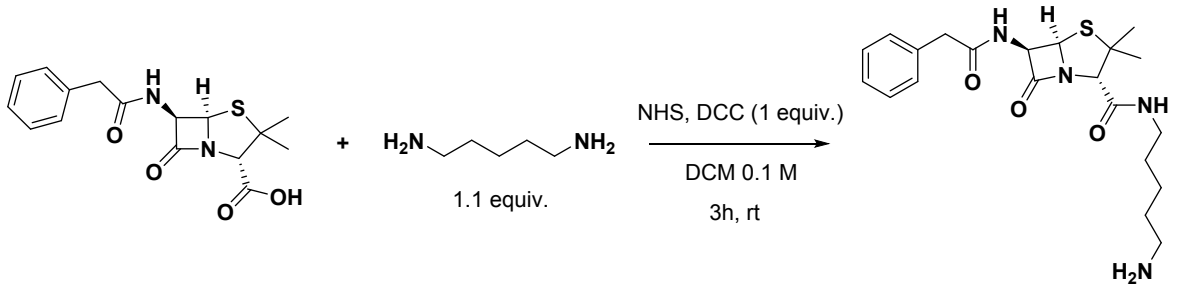
0.165 mmol (1.1 equiv) of the corresponding diamine was added dropwise at 0 ºC under nitrogen atmosphere to 0.150 mmol of the corresponding BLC (the free acid) dissolved in DCM/DMF (0.1 M), treated with NHS and DCC (1 equiv.). The reaction was stirred for 3 h at room temperature. The solution mixture was filtered under vacuum, concentrated and dried under reduced pressure to give a white powder that was characterized by NMR and MS and used without further purification. Haptens **7**-**13** (Scheme S1) were obtained though this route.

*Hapten **7***



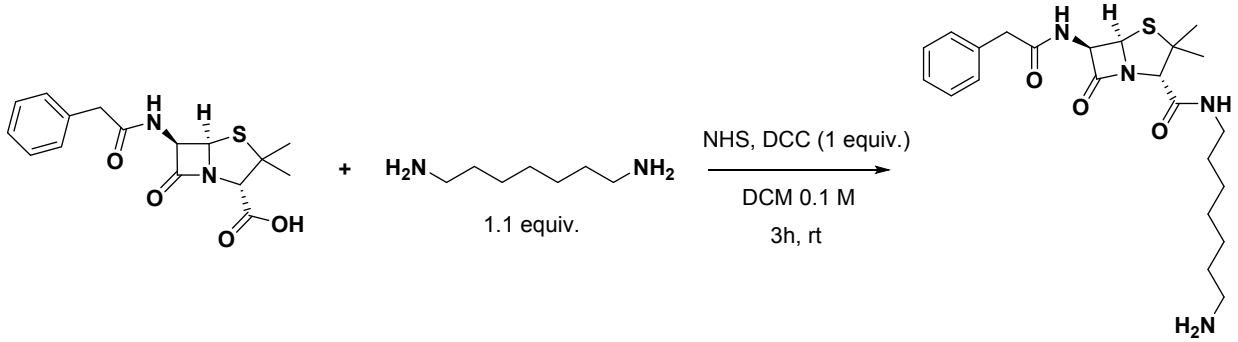
Yield: 100%. HRMS (ESI-TOF) m/z calculated for $C_{19}H_{26}N_4O_3S$ ([M+H$^+$]): 391.1798, found 391.1802.

*Hapten **8***



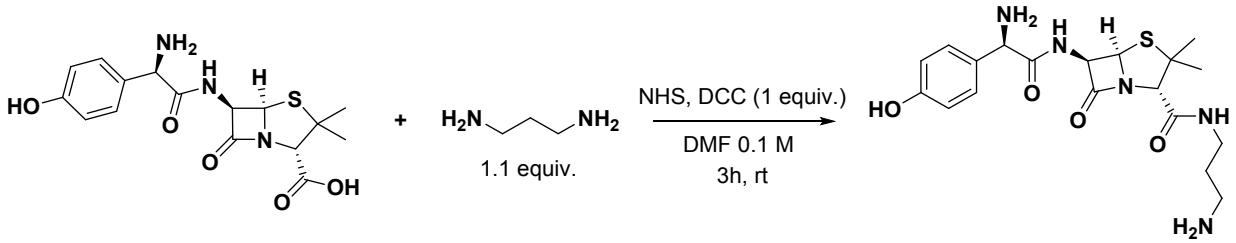
Yield: 66%. HRMS (ESI-TOF) m/z calculated for $C_{21}H_{30}N_4O_3S$ ([M+H$^+$]): 419.2111, found 419.2119.

*Hapten **9***



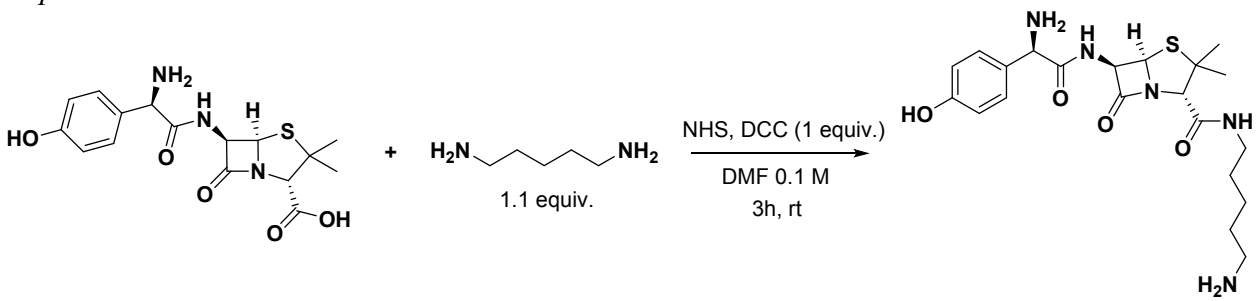
Yield: 77%. HRMS (ESI-TOF) m/z calculated for $C_{23}H_{34}N_4O_3S$ ([M+H$^+$]): 447.2424, found 447.2430.

*Hapten **10***



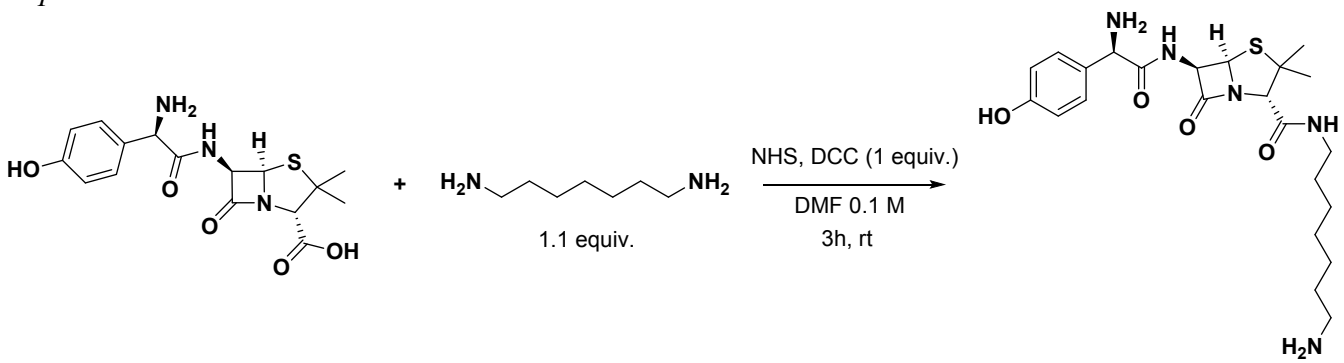
Yield: 51%. HRMS (ESI-TOF) m/z calculated for $C_{19}H_{27}N_5O_4S$ ([M+H$^+$]): 422.1857, found 422.1862.

*Hapten **11***



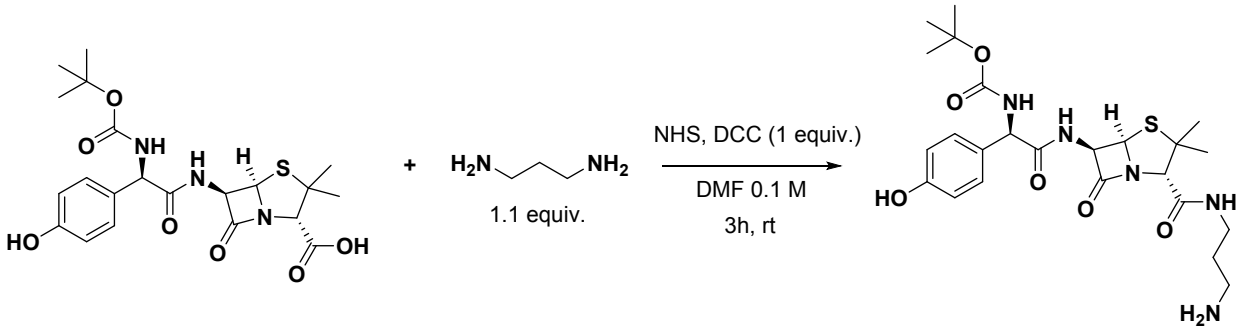
Yield: 14%. HRMS (ESI-TOF) m/z calculated for $C_{21}H_{31}N_5O_4S$ ([M+H$^+$]): 450.2170, found 450.2911.

*Hapten **12***

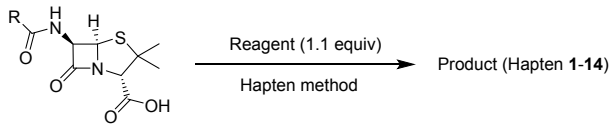


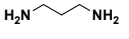
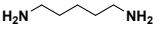
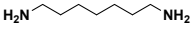
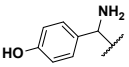
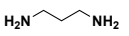
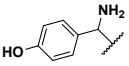
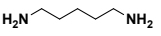
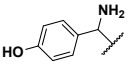
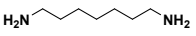
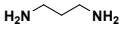
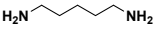
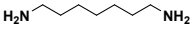
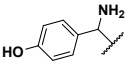
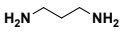
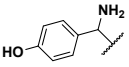
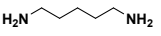
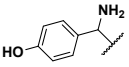
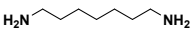
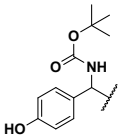
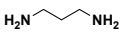
Yield: 81%. HRMS (ESI-TOF) m/z calculated for $C_{23}H_{35}N_5O_4S$ ([M+H$^+$]): 478.2483, found 478.3226.

*Hapten **13***



Yield: 85%. HRMS (ESI-TOF) m/z calculated for $C_{24}H_{35}N_5O_6S$ ([M+H$^+$]): 522.2381, found 522.2561.



Entry	BLC	R	Reagent (1.1 equiv)	Yield[a] (%)	Hapten method[b]
1	PG			97	-oyl
2	PG			61	-oyl
3	PG			77	-oyl
4	AMX			100	-oyl
5	AMX			80	-oyl
6	AMX			100	-oyl
7	PG			100	-anyl
8	PG			66	-anyl
9	PG			77	-anyl
10	AMX			51	-anyl
11	AMX			14[c]	-anyl
12	AMX			81	-anyl
13	AMX			85	-anyl
14	PG		-		Reference

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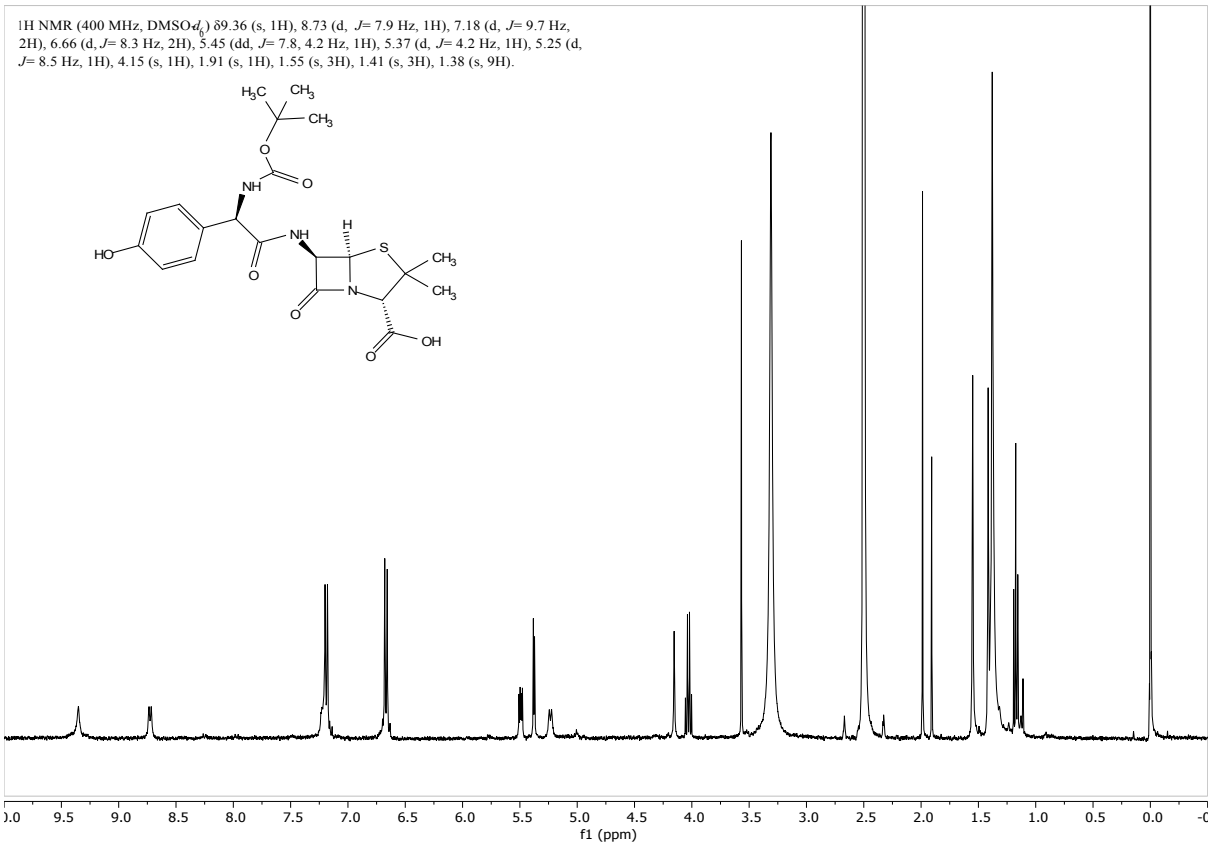
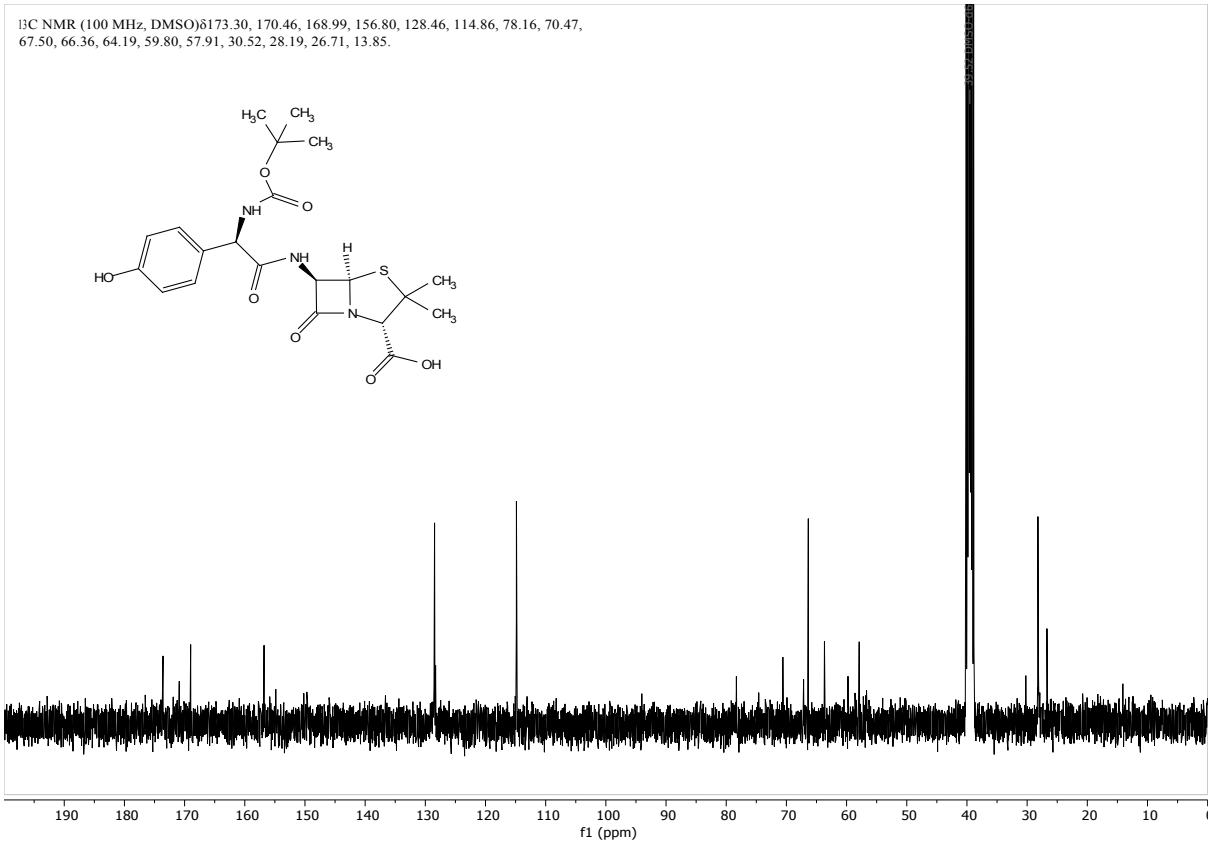
187 Scheme S1: Chemical structures of the BLC synthetized haptens using alkyl diamines. [a]
188 Isolated yields were reported. [b] Reaction conditions: (I) -oyl haptens: Na_2CO_3 0.5 M, pH
189 11.0, then HCl 6.0 M, pH=4.0, 3h, rt; (II) -anyl haptens: DCM/DMF 0.1 M, 3h, rt. [c] Poor
190 yield due to transfer loses.

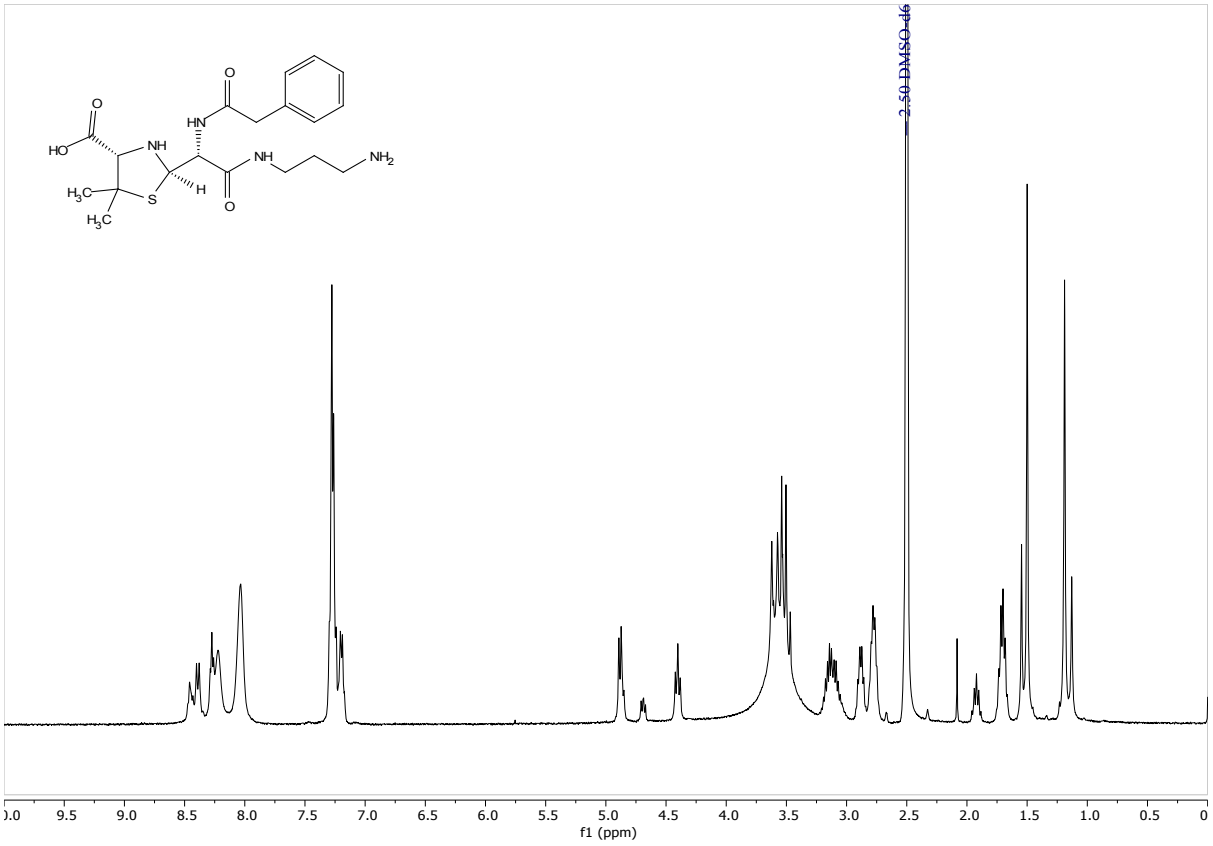
4. NMR spectra

Boc-amoxicillin





195 *Hapten **1***



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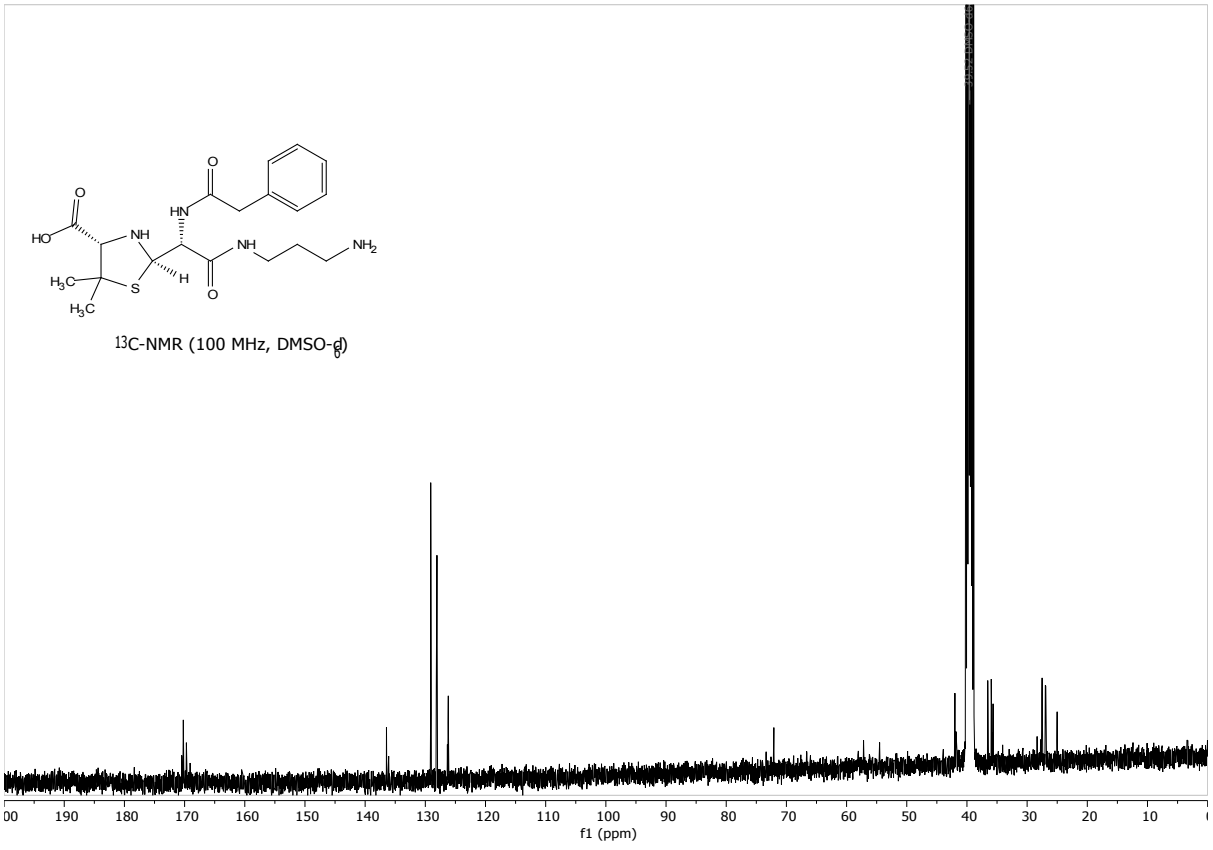

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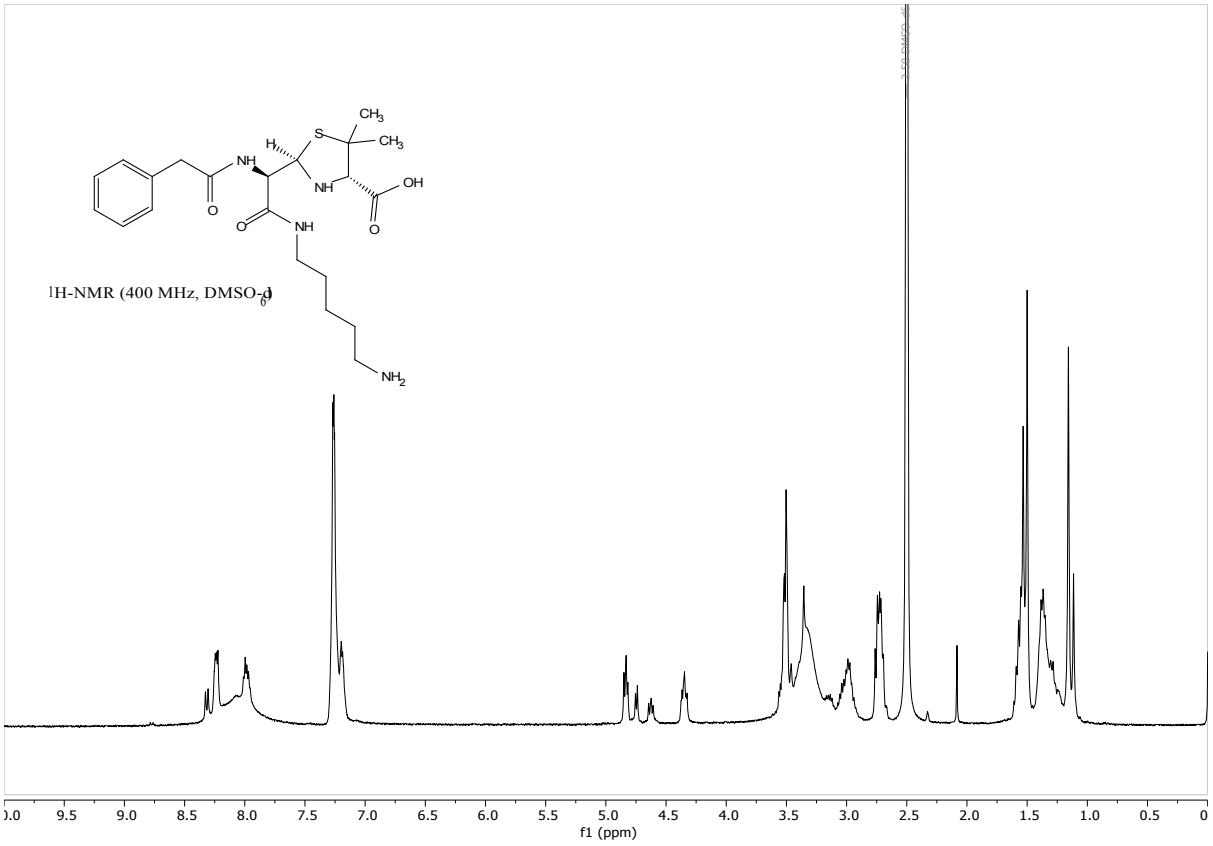

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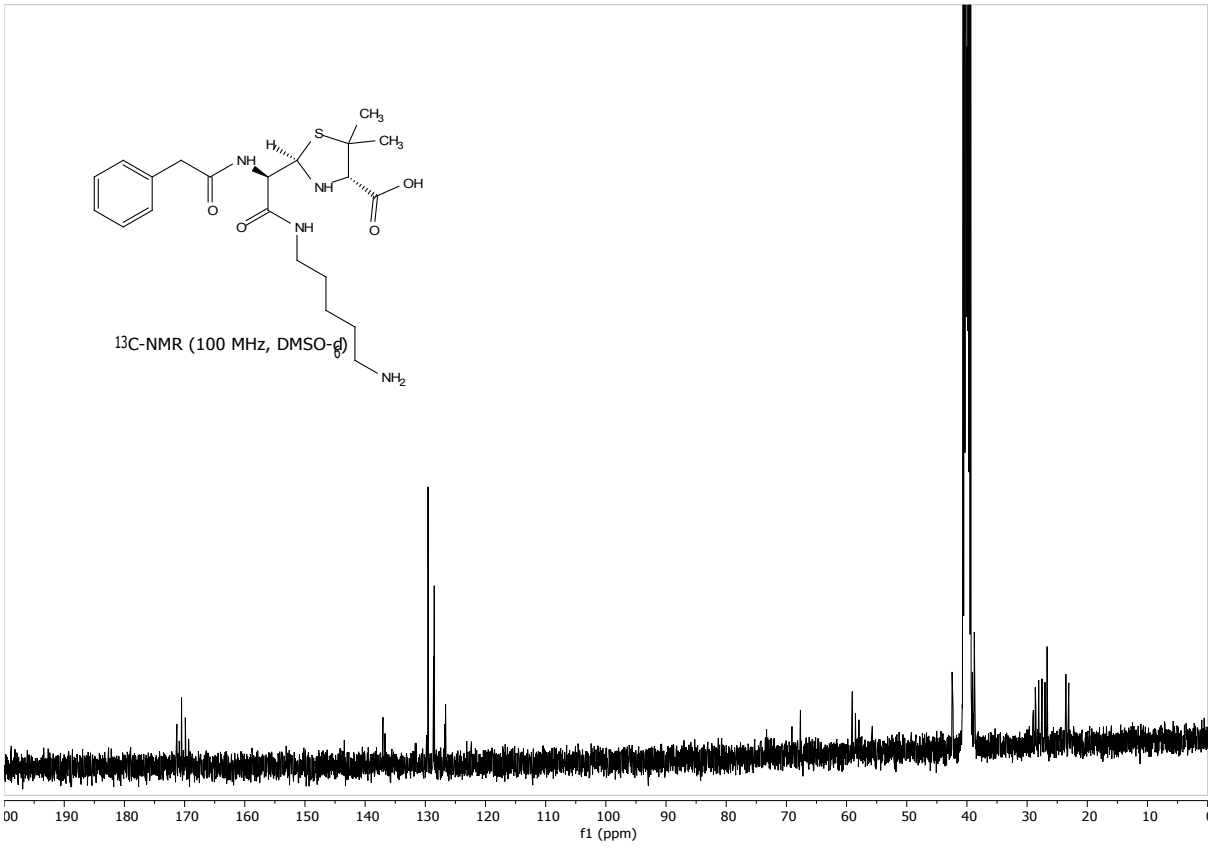

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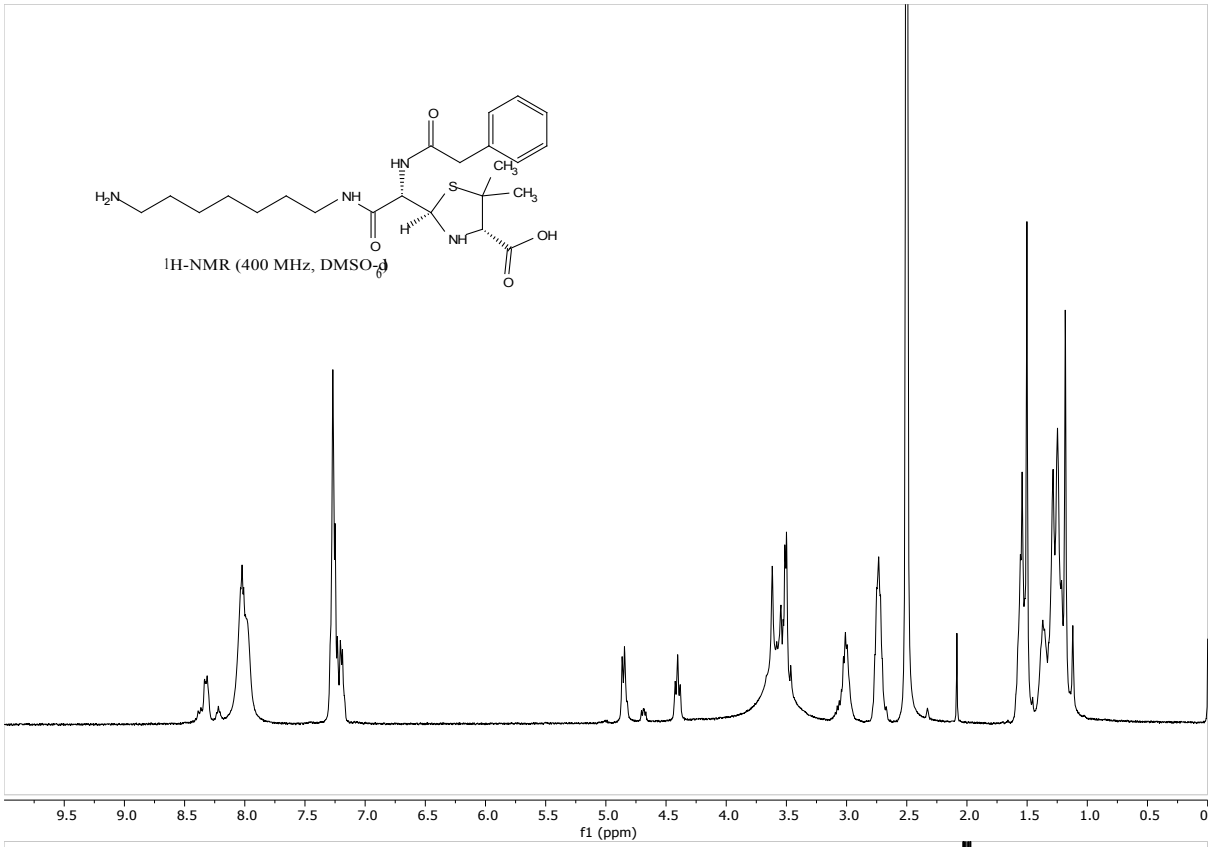

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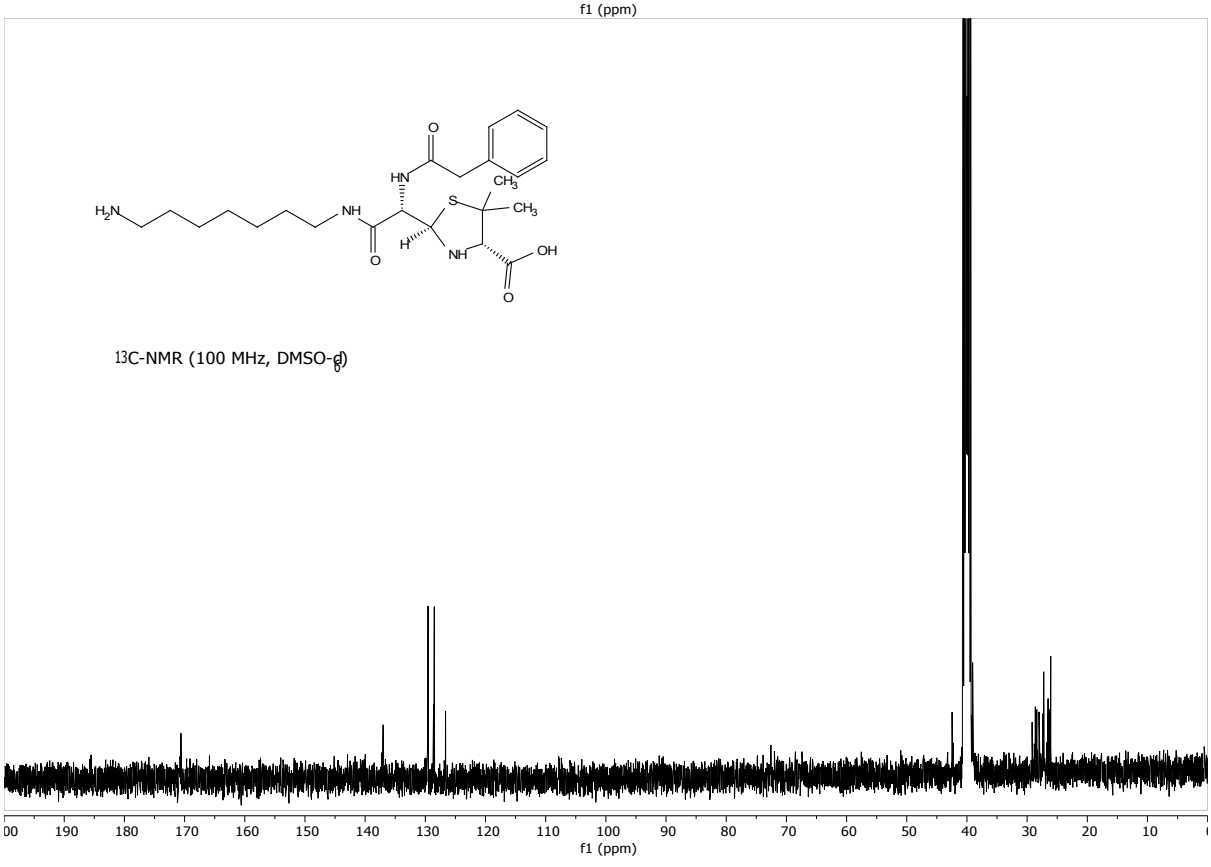

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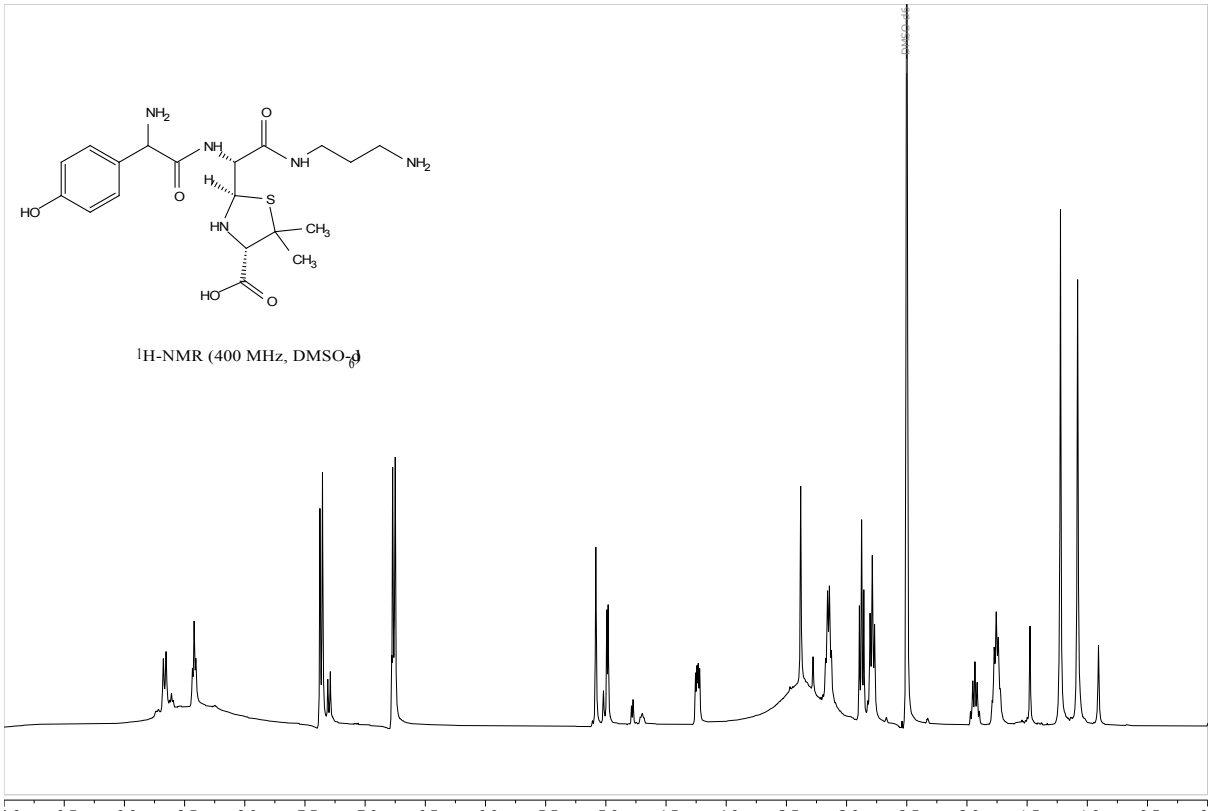

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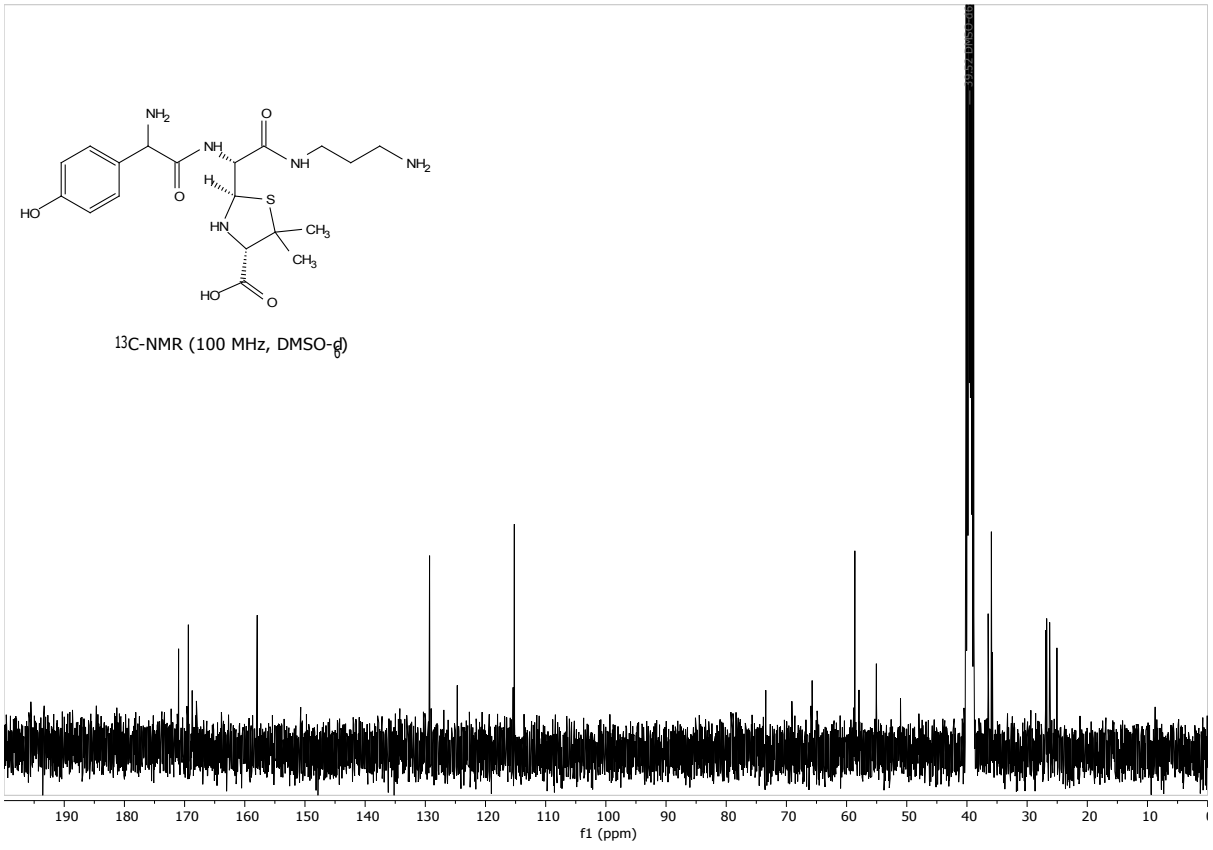

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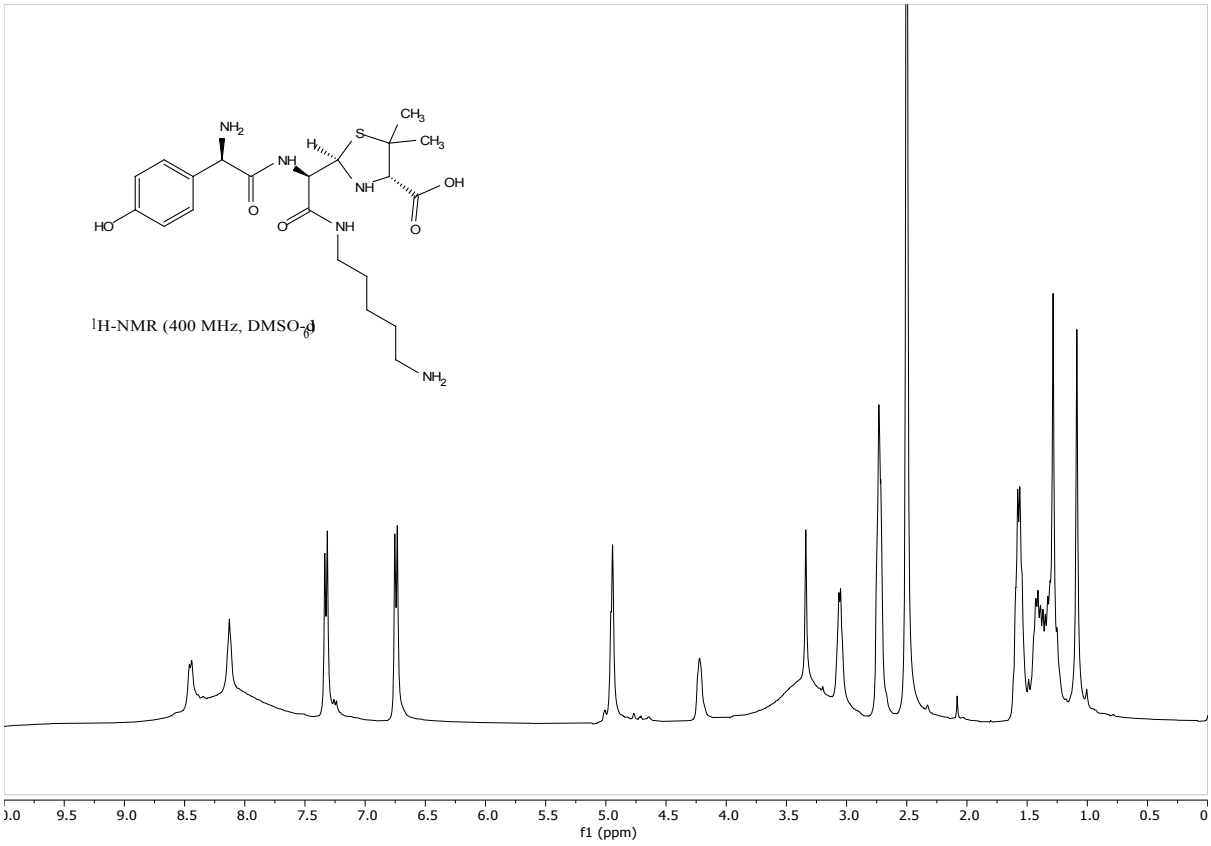

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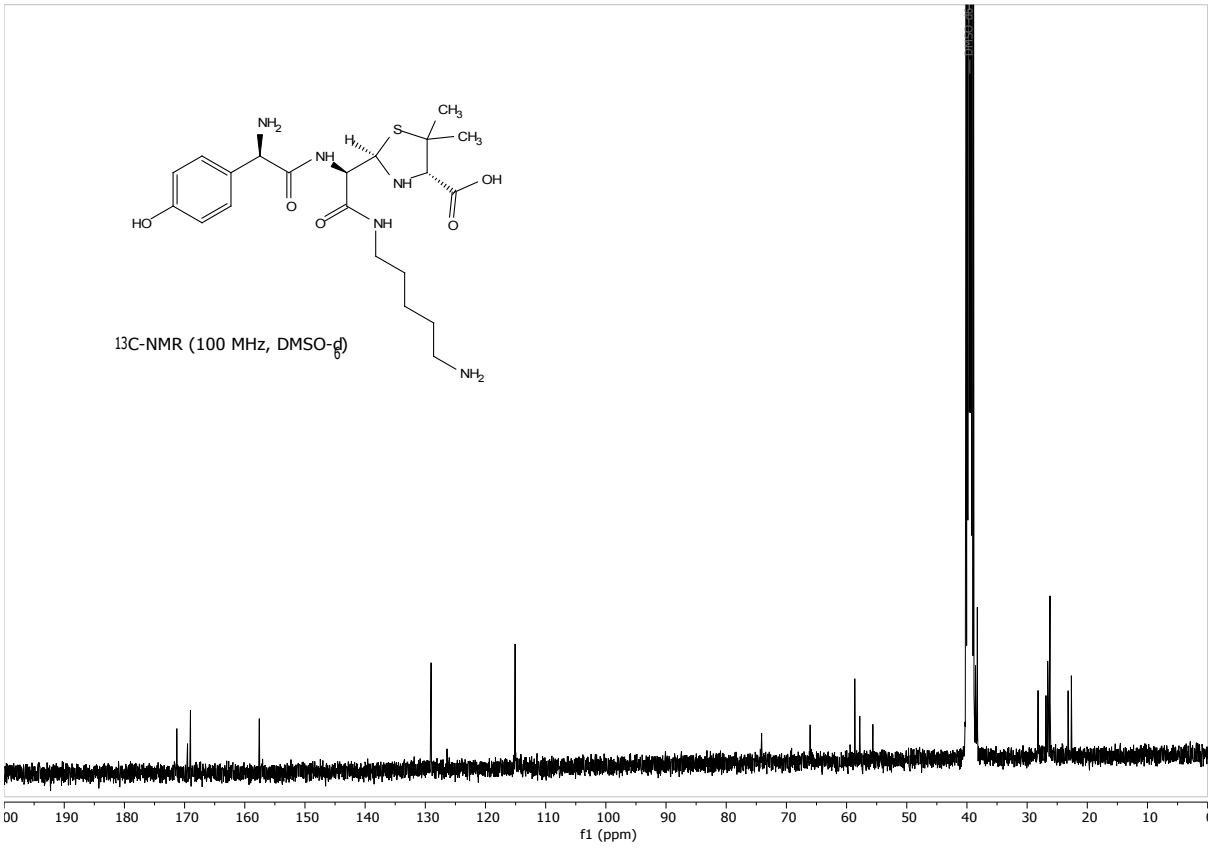

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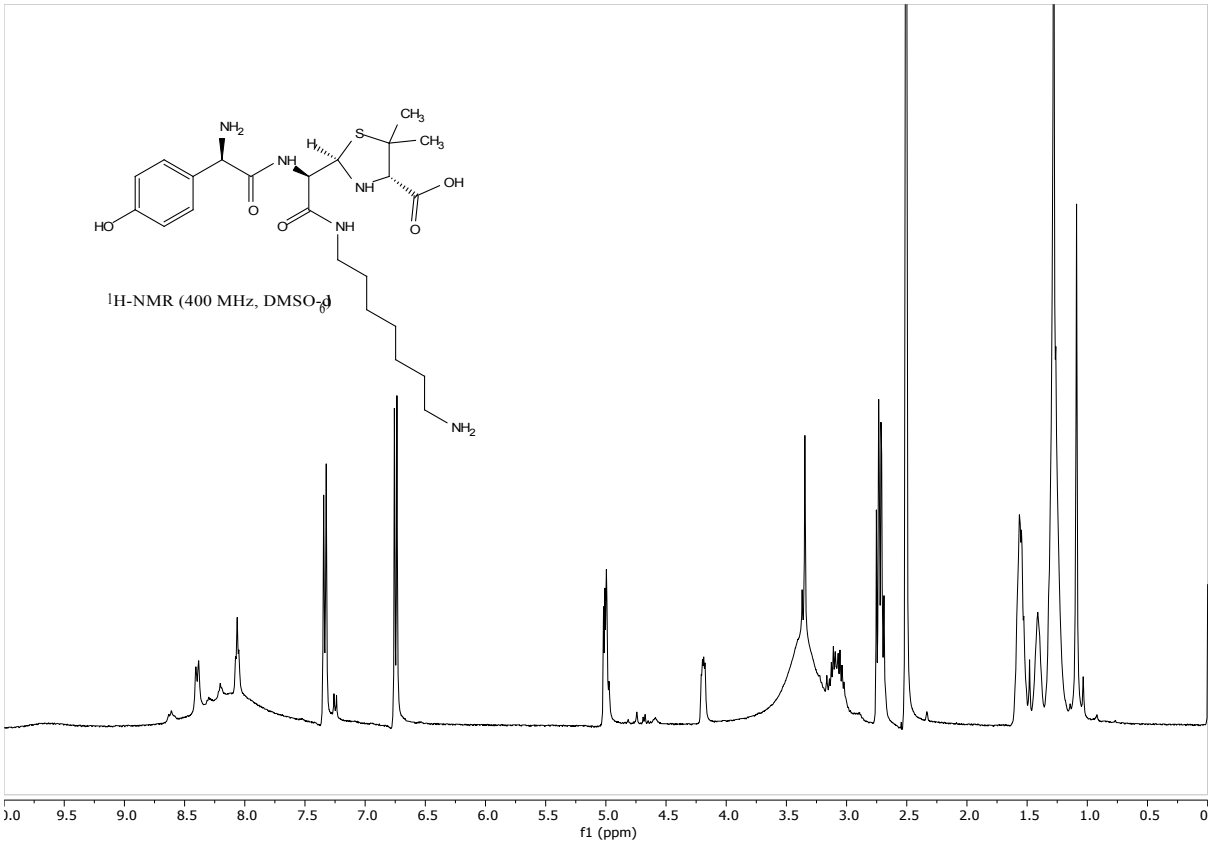

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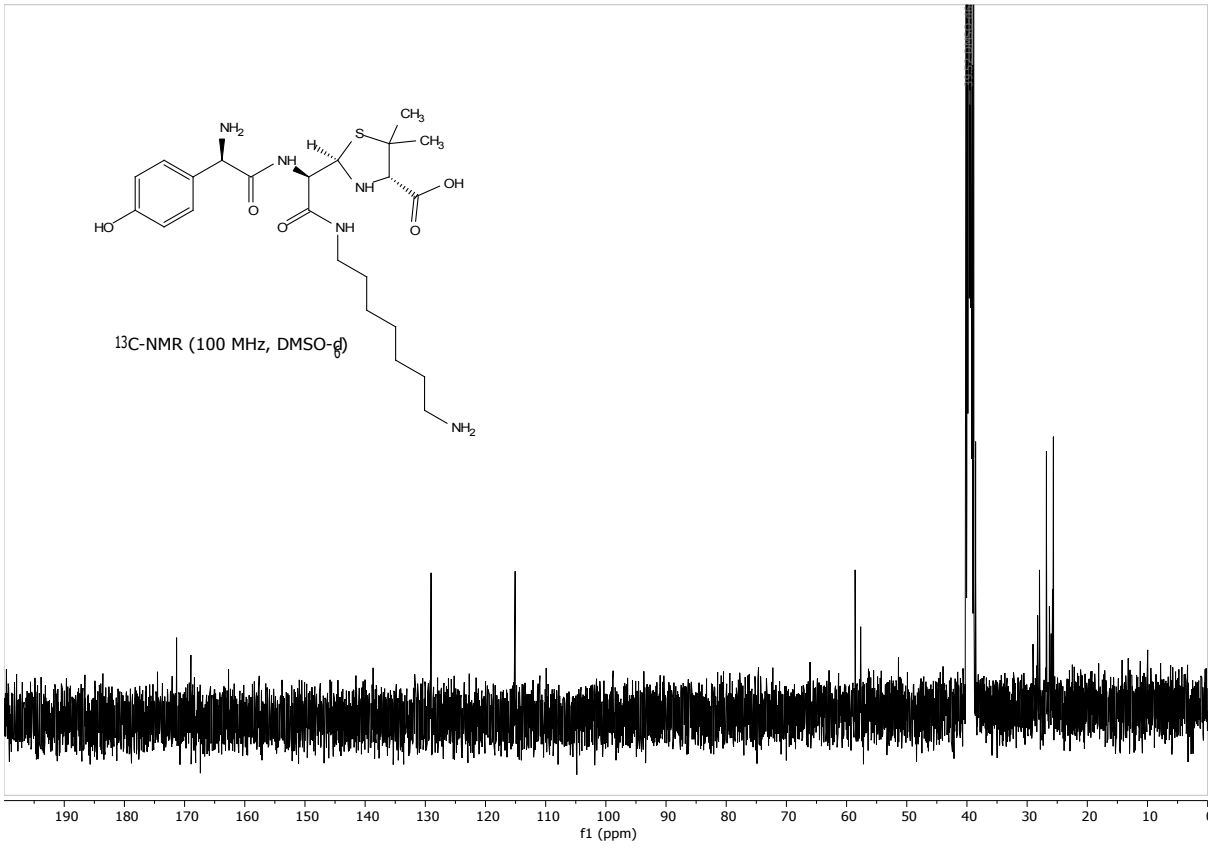

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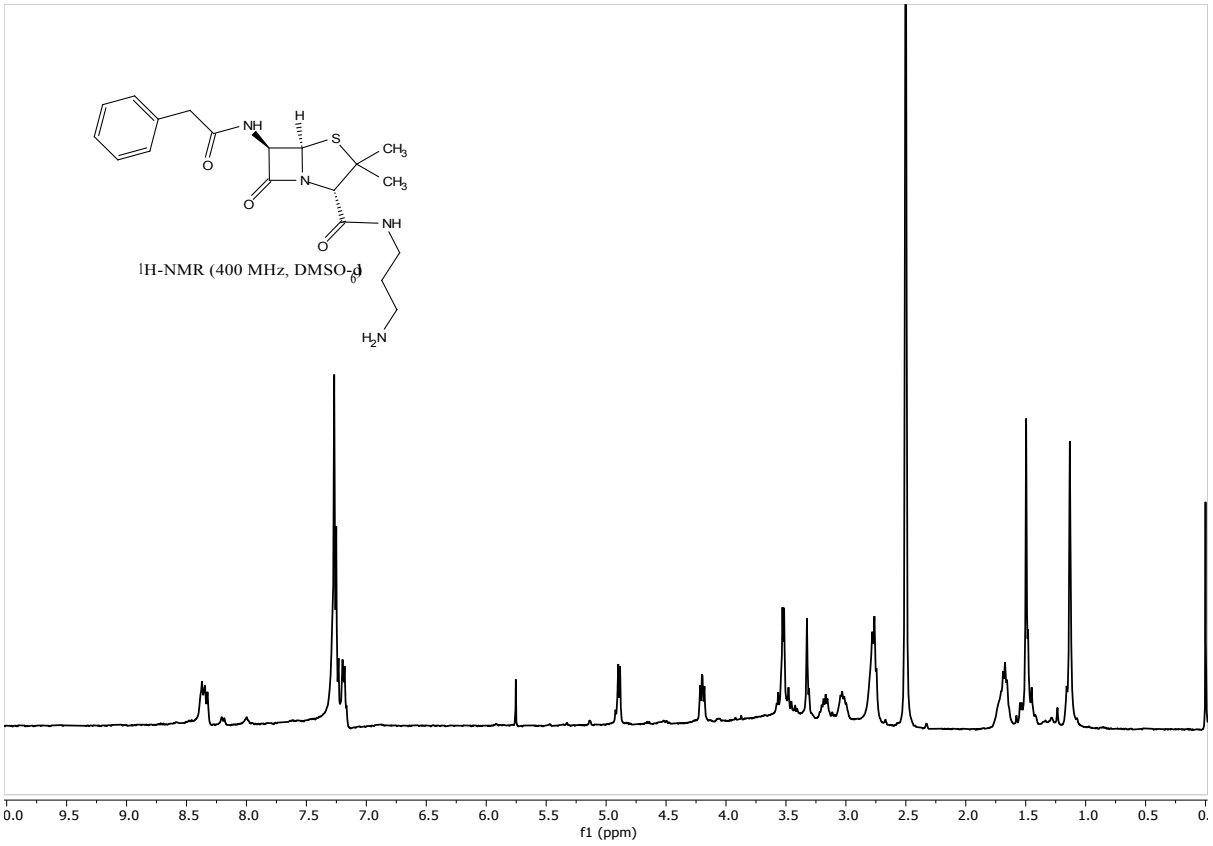

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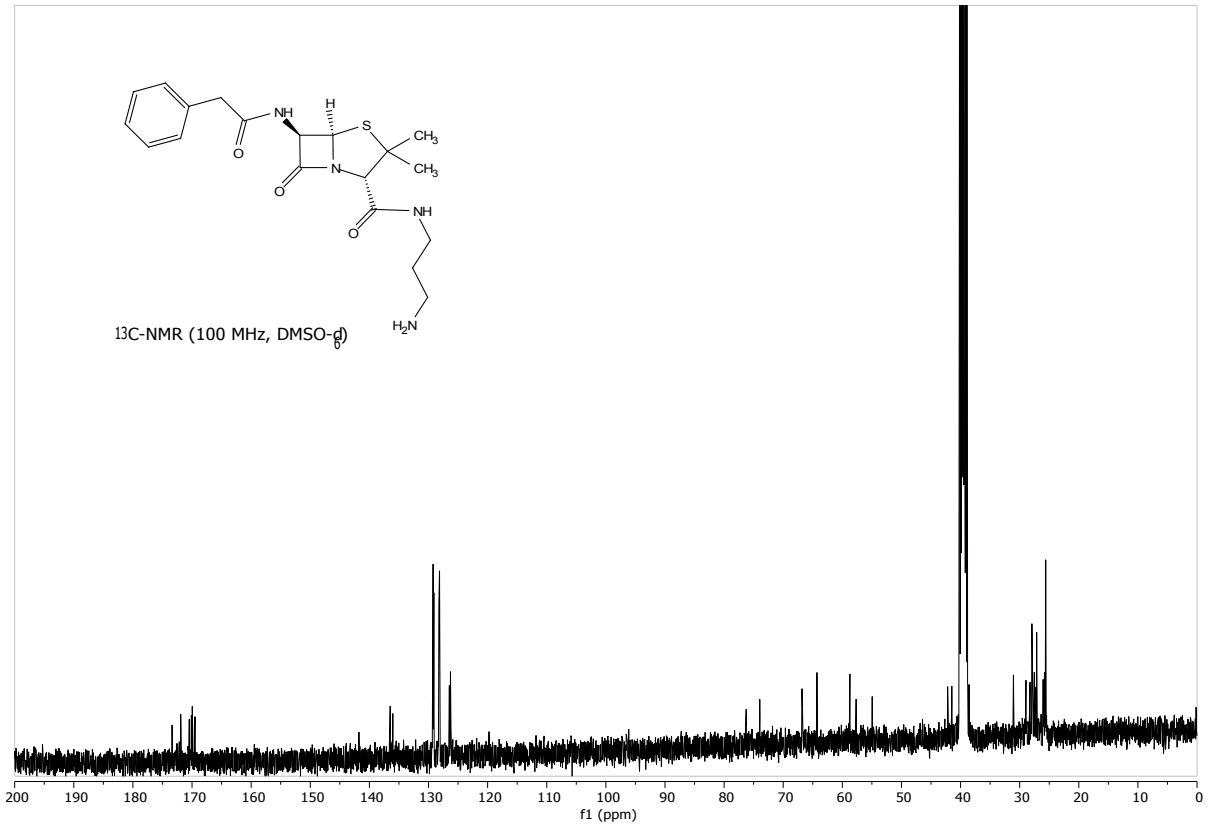

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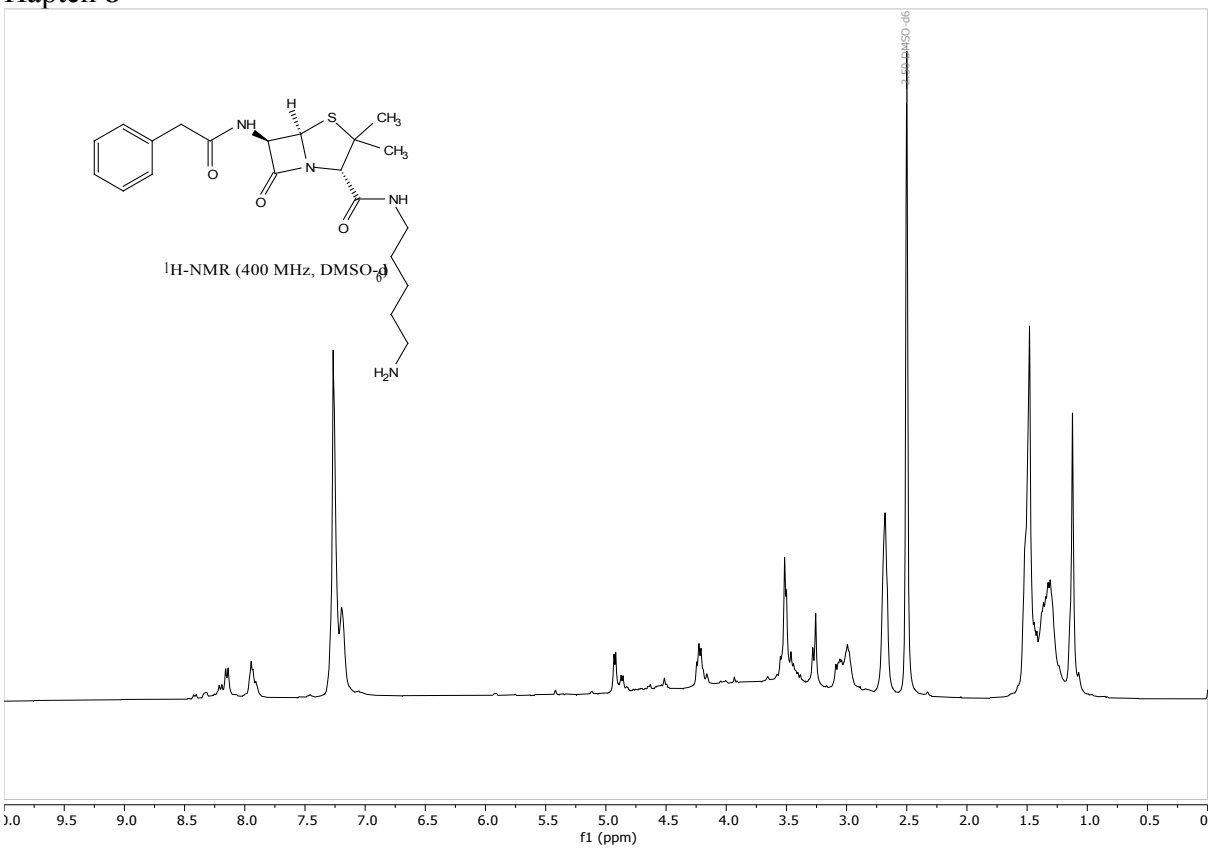

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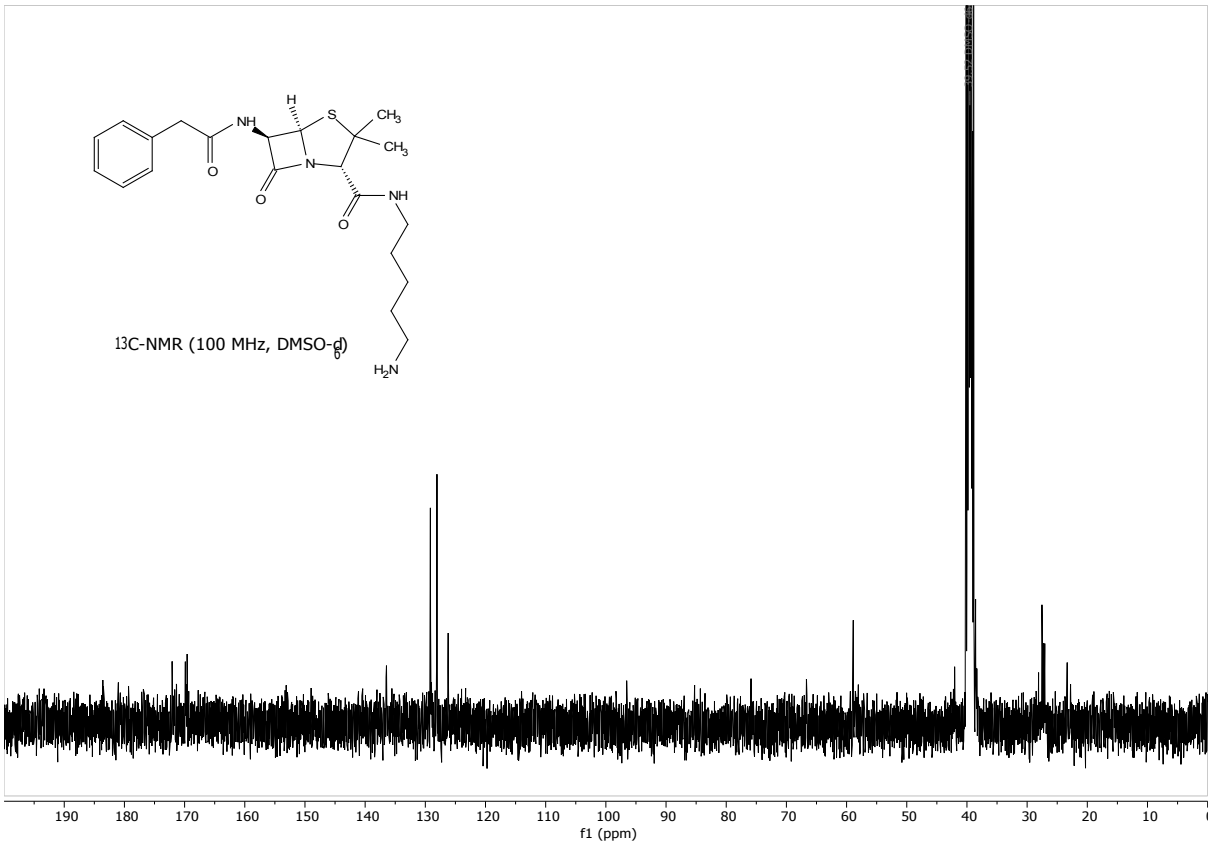

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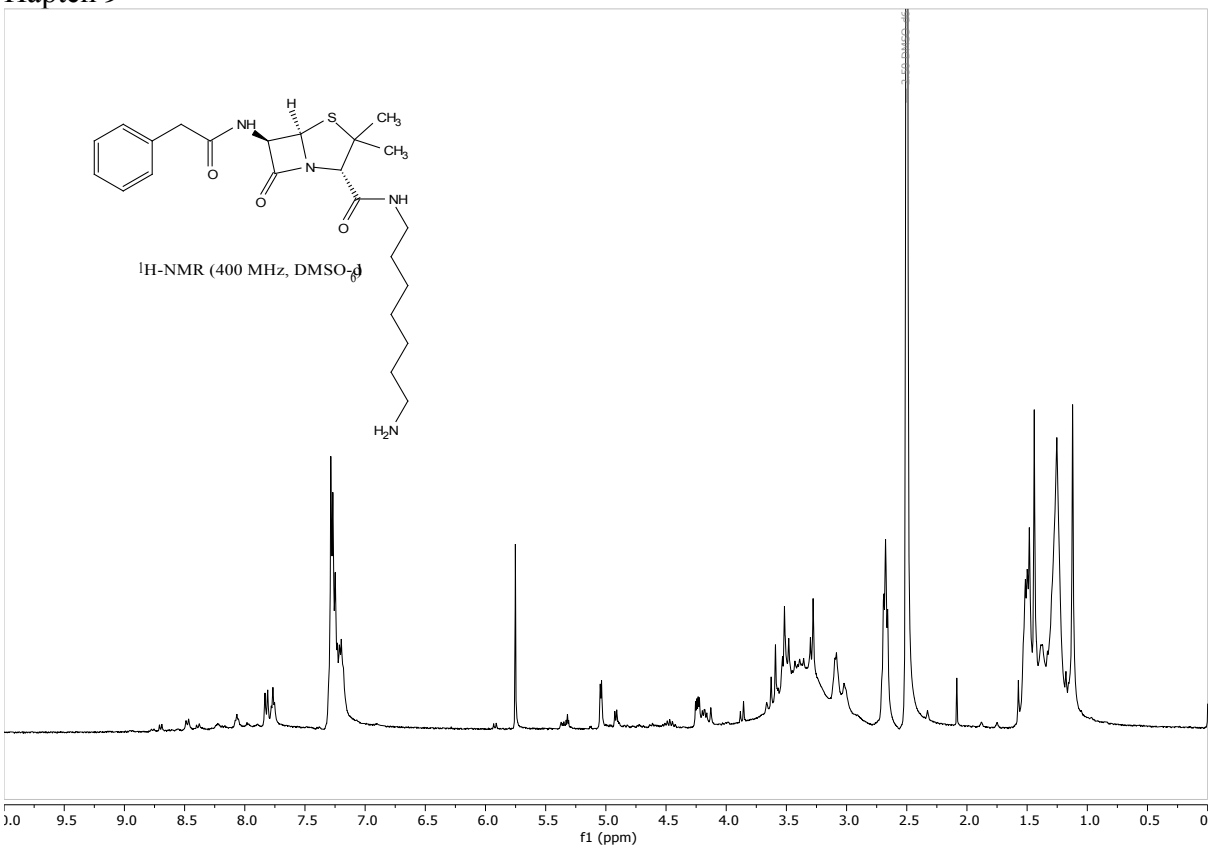

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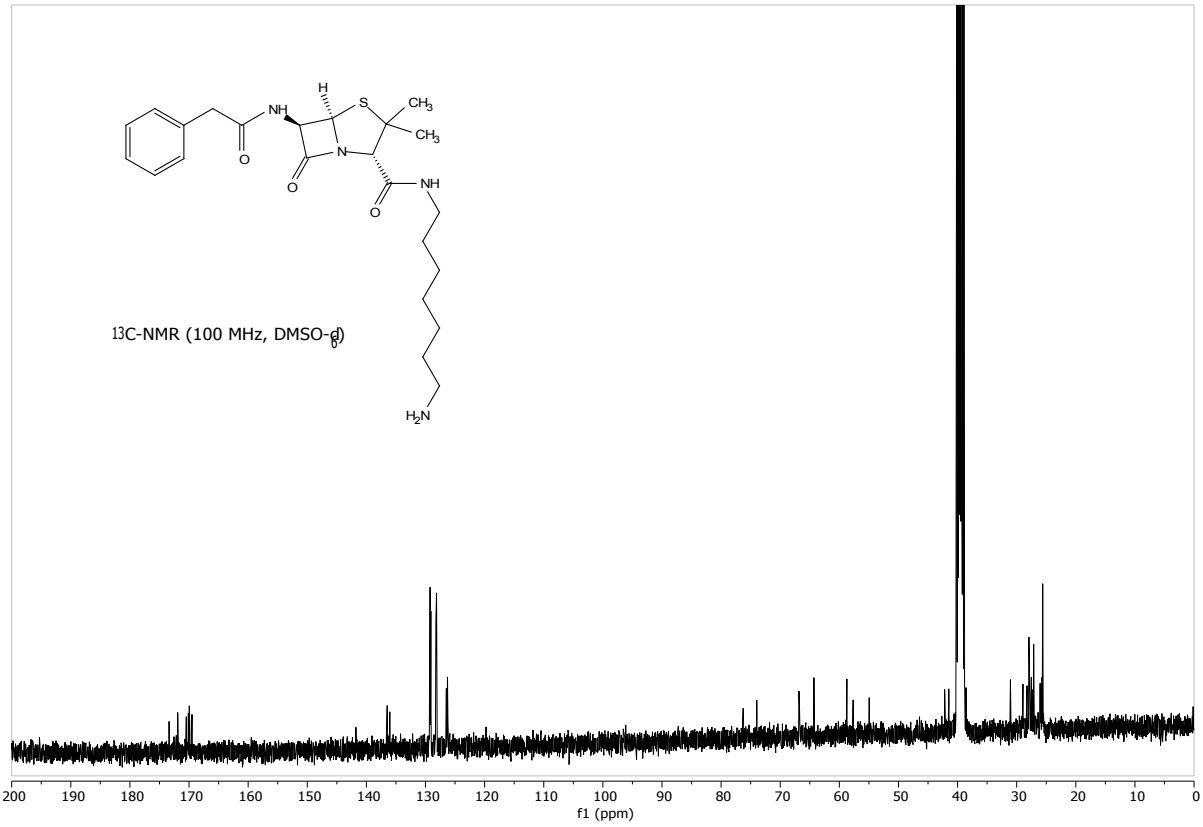

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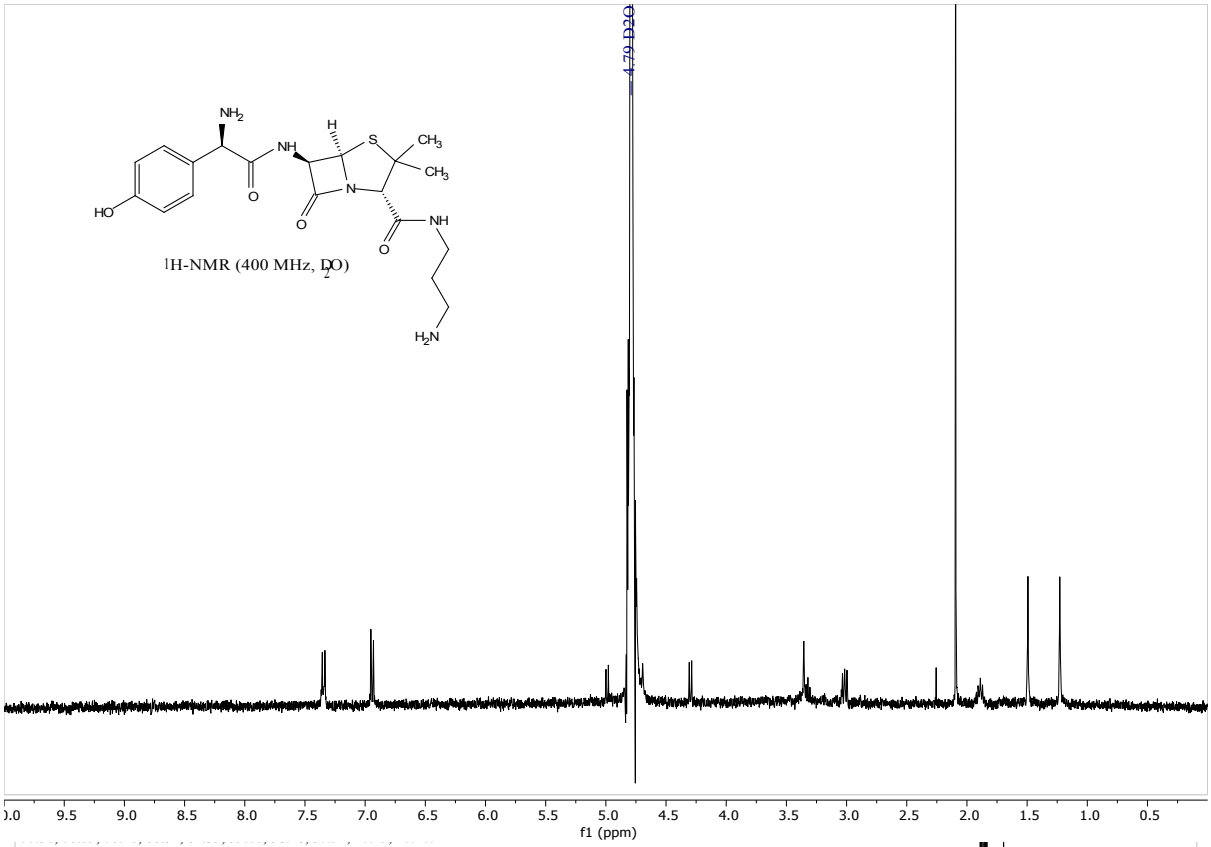

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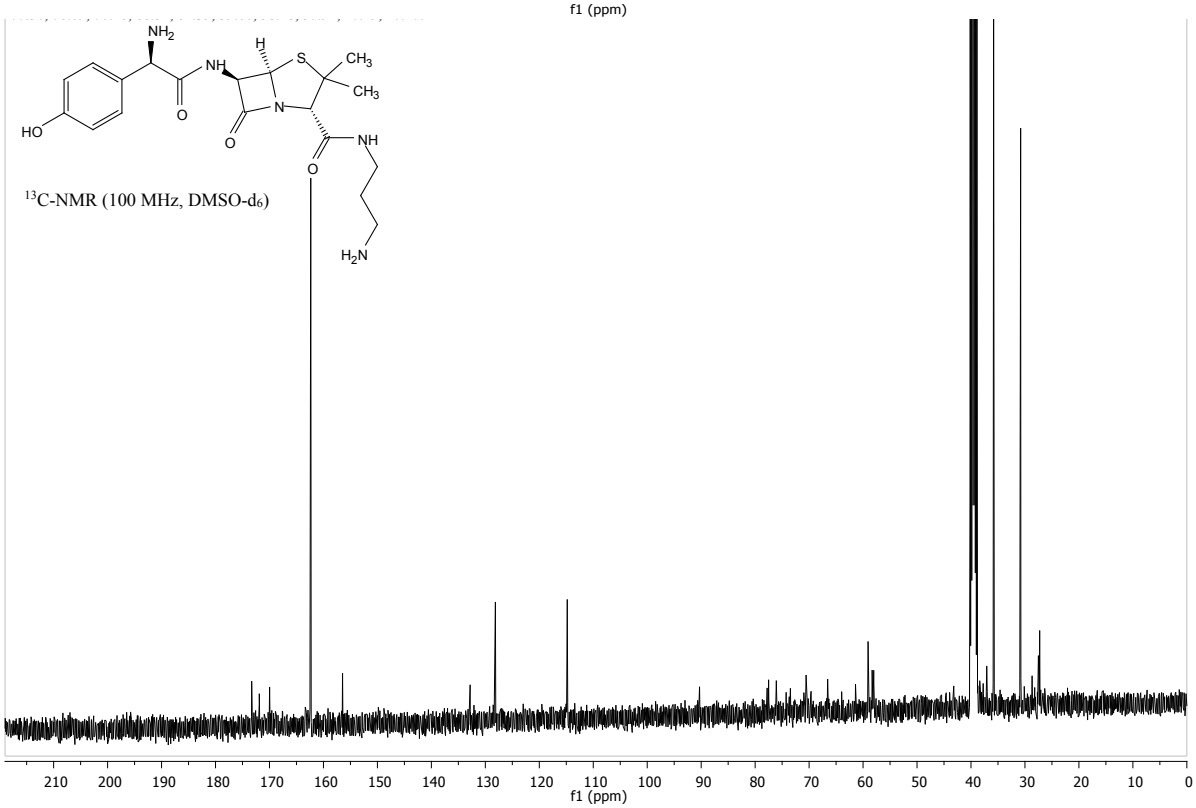

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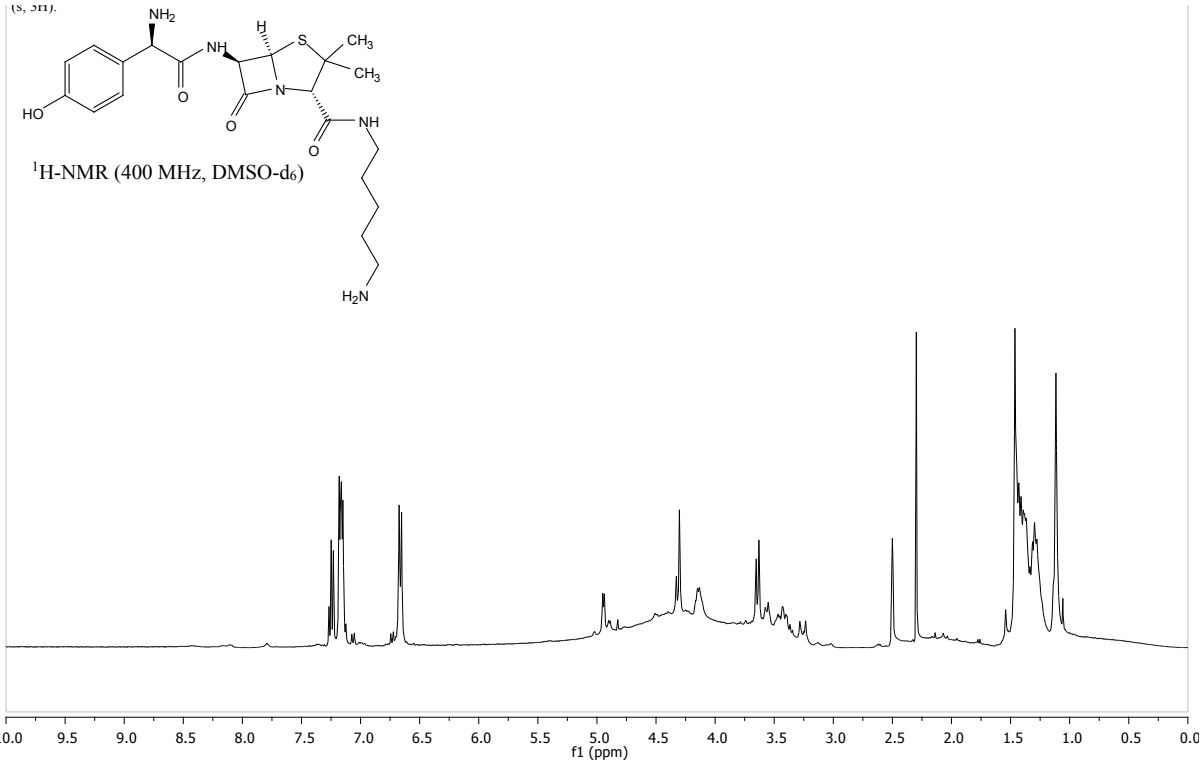

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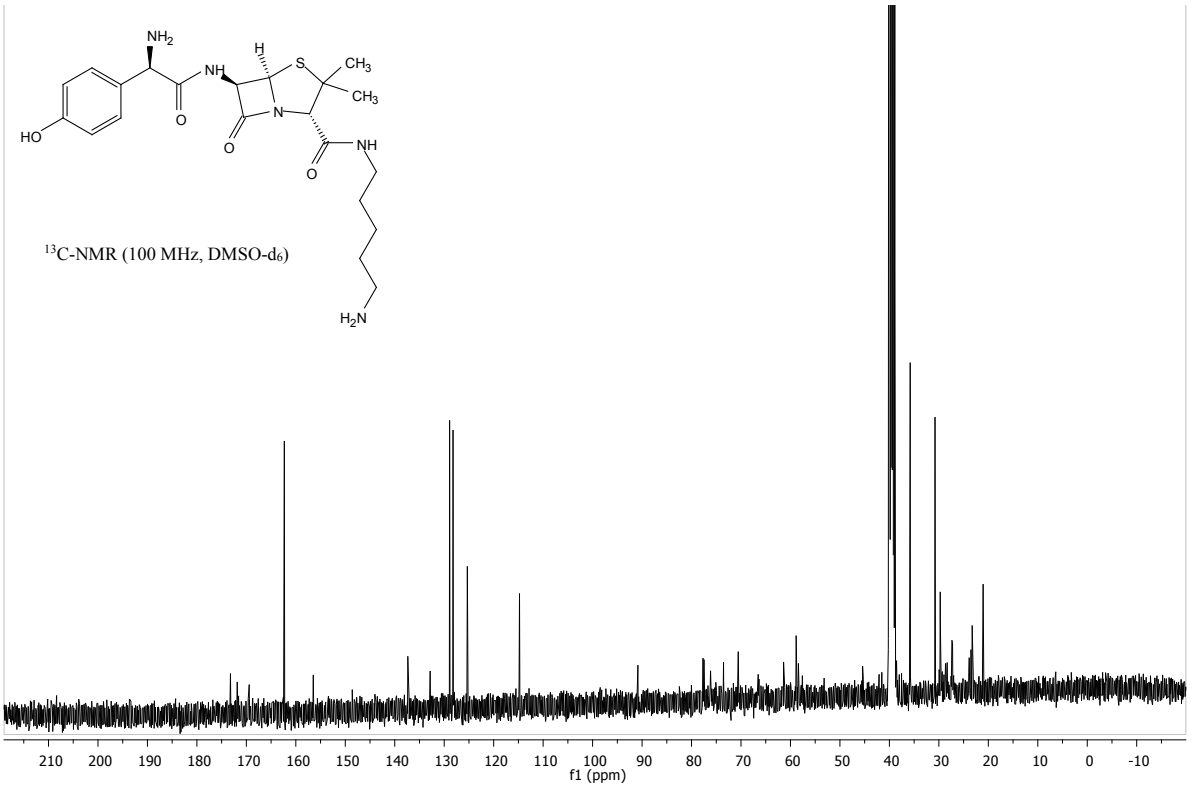

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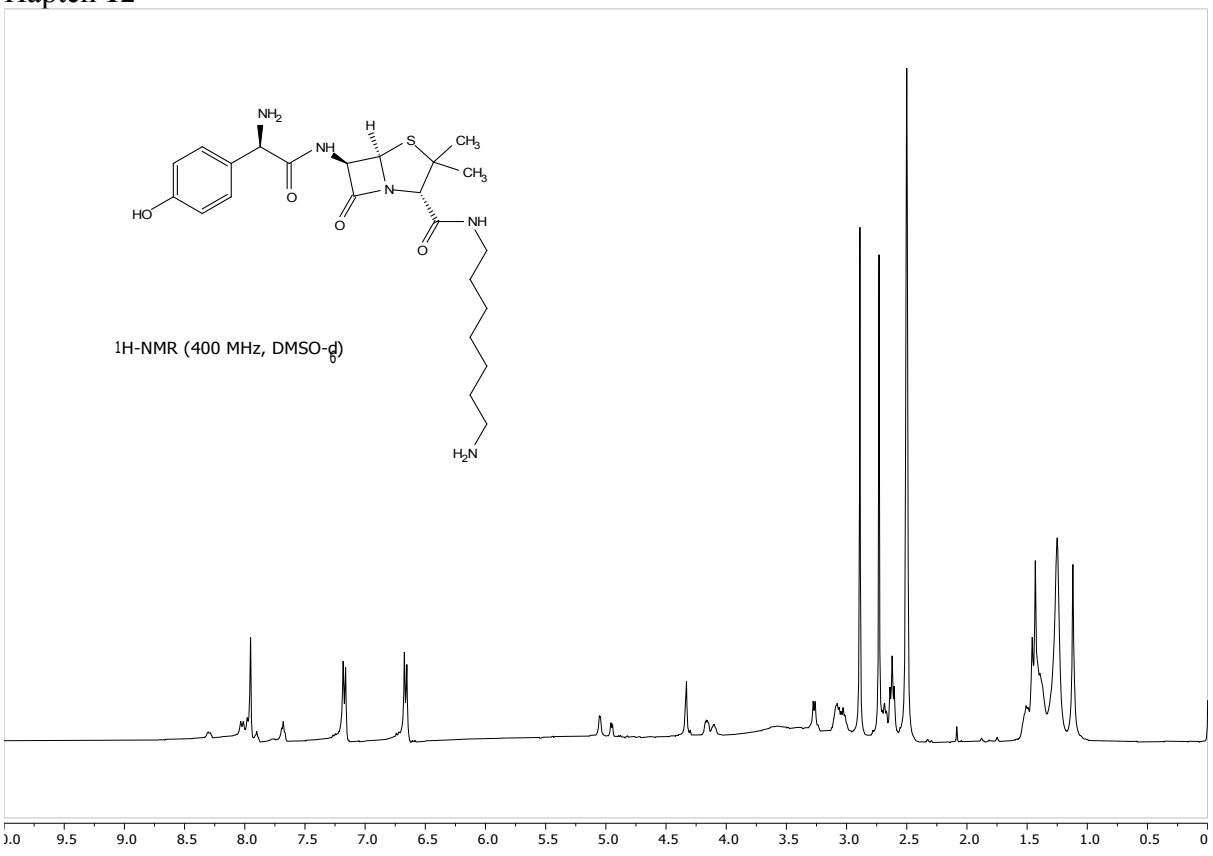

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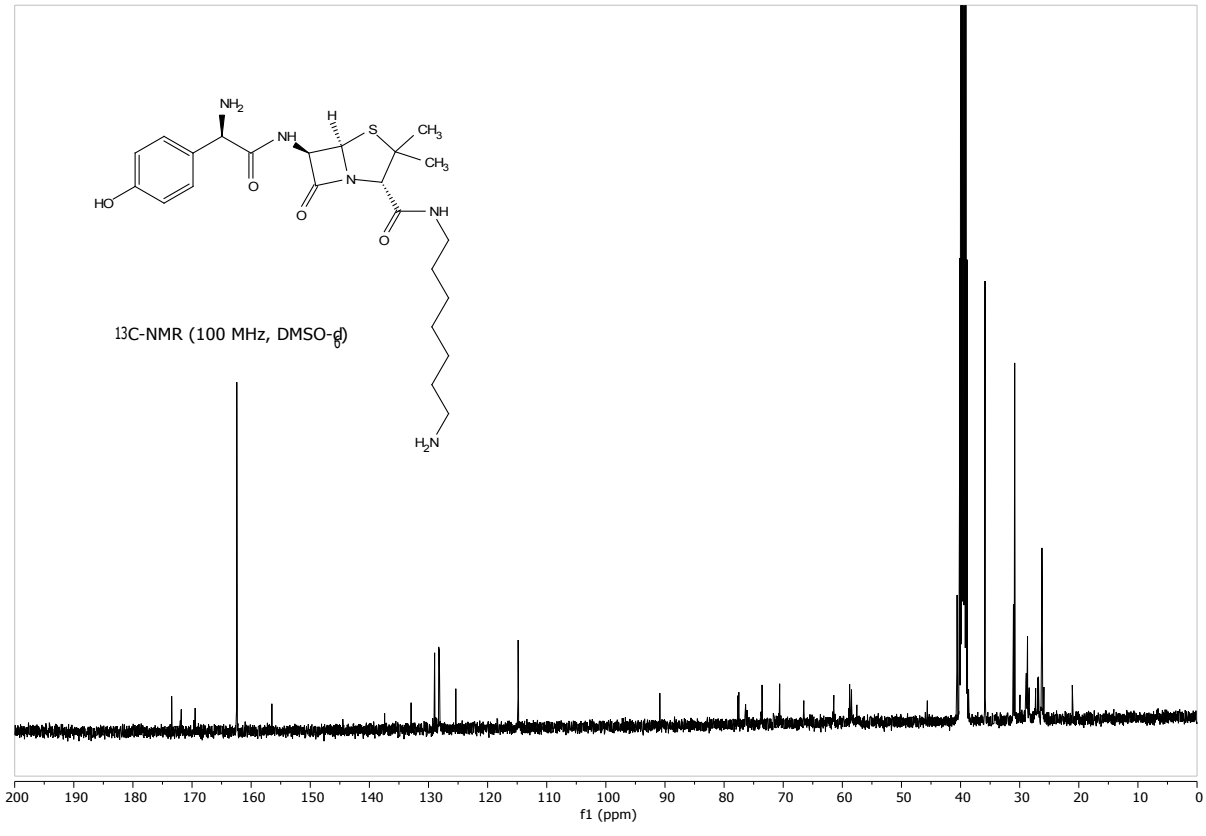

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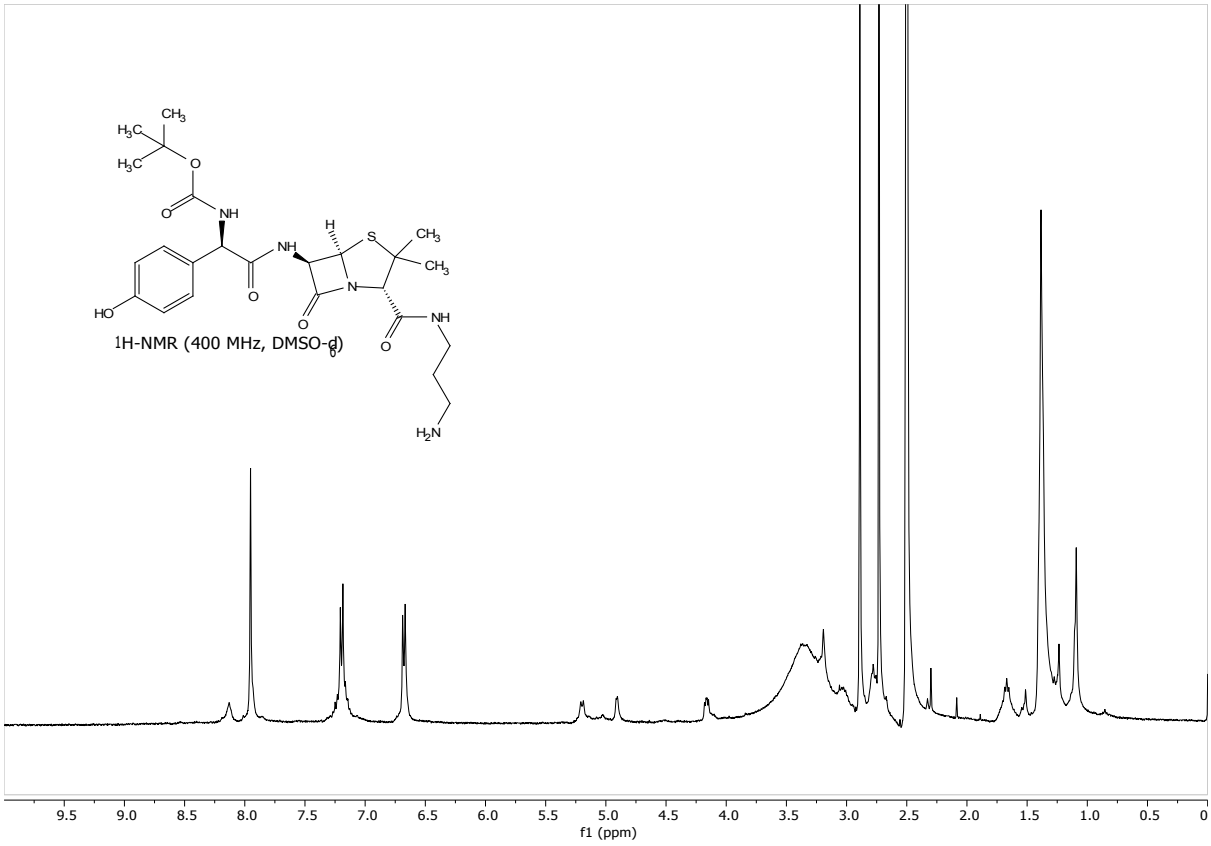

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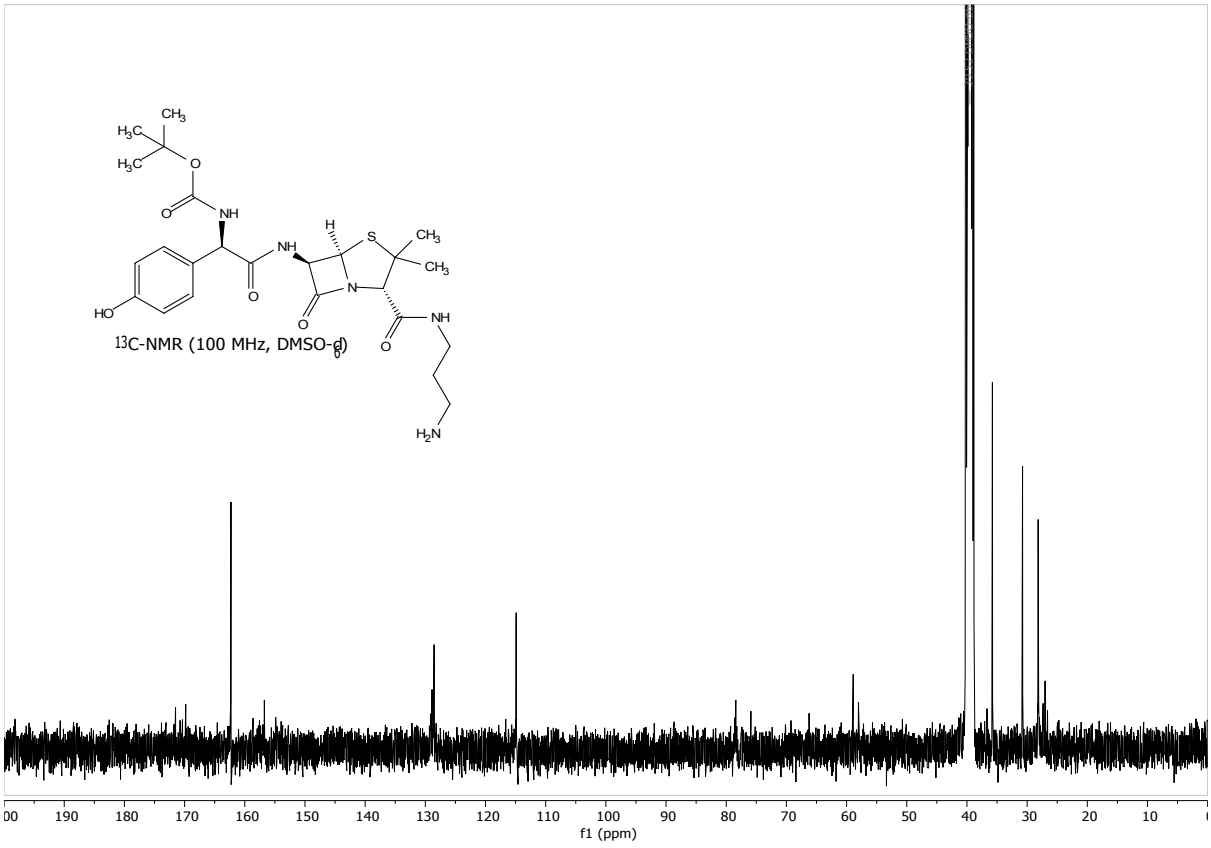
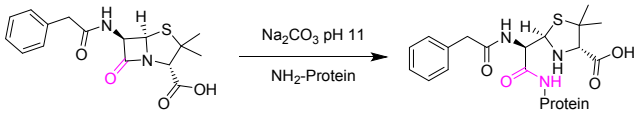


233

5. Preparation of the antigens

Strategy I) Using synthesized haptens

In the case of the PG reference antigen (Scheme S1, entry **14**) and the negative control (AZT), the conjugation was performed through the lysine residues of the protein HSA by β-lactam ring opening, through amidation between the carbonyl carbon of the β-lactam ring and amino group of lysine residues (Scheme S3), as previously described[1] with few modifications. Briefly, HSA (2.0 mg) dissolved in sodium carbonate 0.5 M, pH 11.0, reacted with PG or AZT (0.03 mmol, 1000 equiv.) overnight at room temperature.



Determinant 14
(Reference)

Scheme S3: PG-oyl formation after β-lactam ring opening.

Modified -oyl and –anyl antigens were prepared following the carbodiimide chemistry. For that, HSA (2.0 mg) and the corresponding impure modified BLC hapten (4.0 mg) were dissolved in 200 µL and 1 mL, respectively, of sodium phosphate buffer 0.1 M, NaCl 0.15 M, pH=7.2. Then, 500 µL of the BLC hapten solution was mixed up in an Eppendorf with the HSA solution (200 µL) and EDC (10.0 mg) was added. The solution was allowed to react at room temperature for 2 hours.

All antigens were purified by gel filtration chromatography on Amicon Ultra 0.5 pre-concentred 10 K filters using PBS 1X, pH 7.4, as elution buffer. Finally, they were diluted to 1.0 mg/mL and stored at -20 ºC until used. Protein quantification was performed by the Coomassie/Bradford[2] colorimetric assay. Molar ratios protein-hapten were determined by MS-MALDI-TOF[3].

Strategy II) Using cationized carrier molecules

Diamine dihydrochloride salts used in this study were ethylene diamine, 1,4-diaminobutane and 1,4-phenylenediamine. Major antigens were produced with this strategy and the carrier proteins used were HSA and H1.

In order to cationize carrier proteins, 1.0 mmol of the corresponding diamine dihydrochloride salt (1 equiv) dissolved in 600 µL of MES 0.1 M, pH 4.7 (coupling buffer) was mixed up with 2.0 mg of the carrier protein dissolved in 200 µL of coupling buffer. Then, an EDC hydrochloride solution of 10 mg/mL in coupling buffer was prepared and 200 µL of this solution was added to the reaction mixture. The solution was allowed to react overnight at room temperature. Cationized proteins were purified by gel filtration chromatography on 10 K preconcentred filters using sodium carbonate 0.5 M, pH 11.0, as the elution buffer for the following conjugation step.

Then, a solution of the corresponding BLC (1,000 equiv.) in sodium carbonate 0.5 M, pH 11.0, was added to the solutions. The reactions were allowed to stir at room temperature overnight. Purification and quantification of the protein-modified antigens were performed as explained in Strategy I.

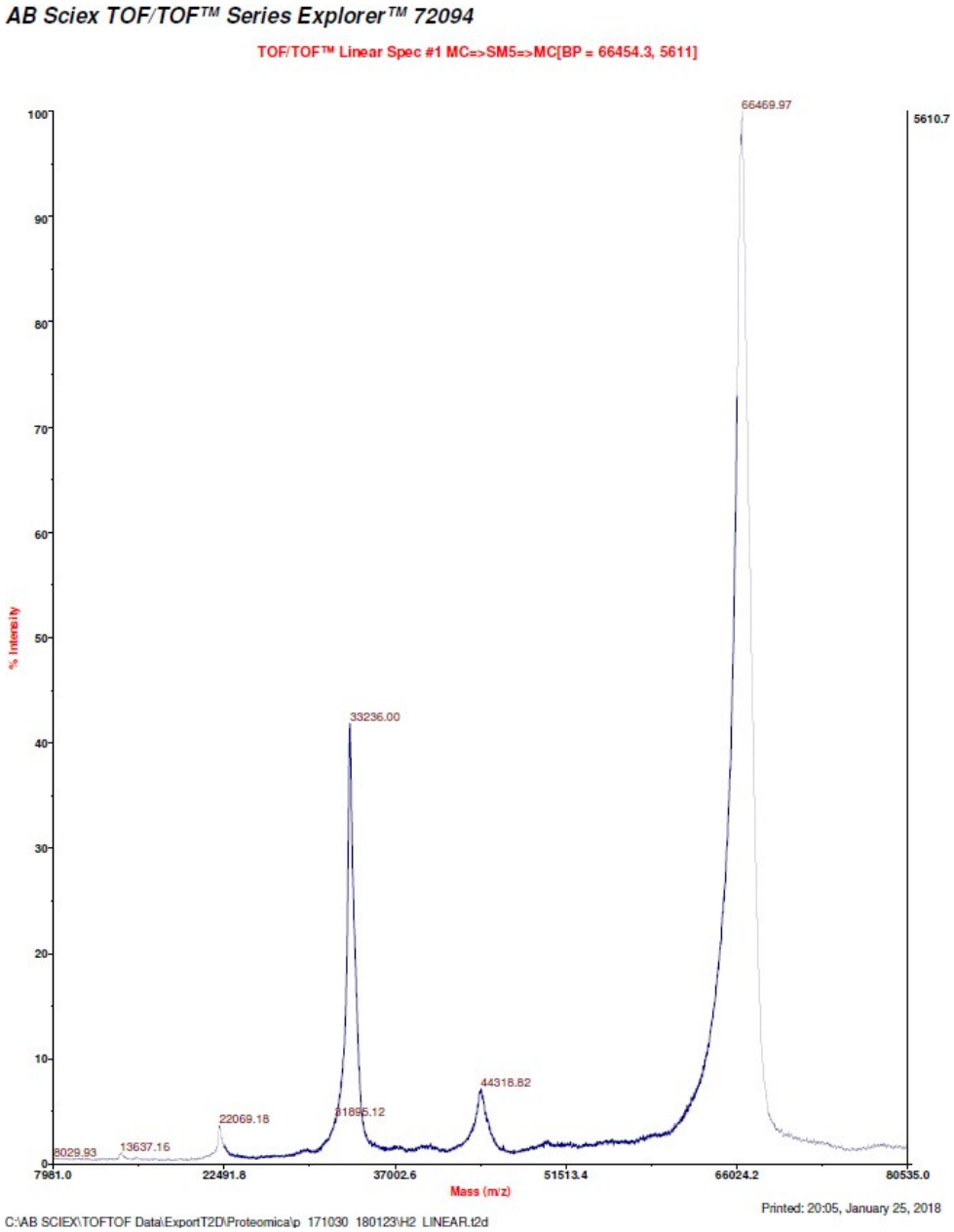
6. MS-MALDI-TOF spectra

The molecular weight (MW) of each antigenic determinant was calculated from the peak centroid of the peaks according to the following equation:

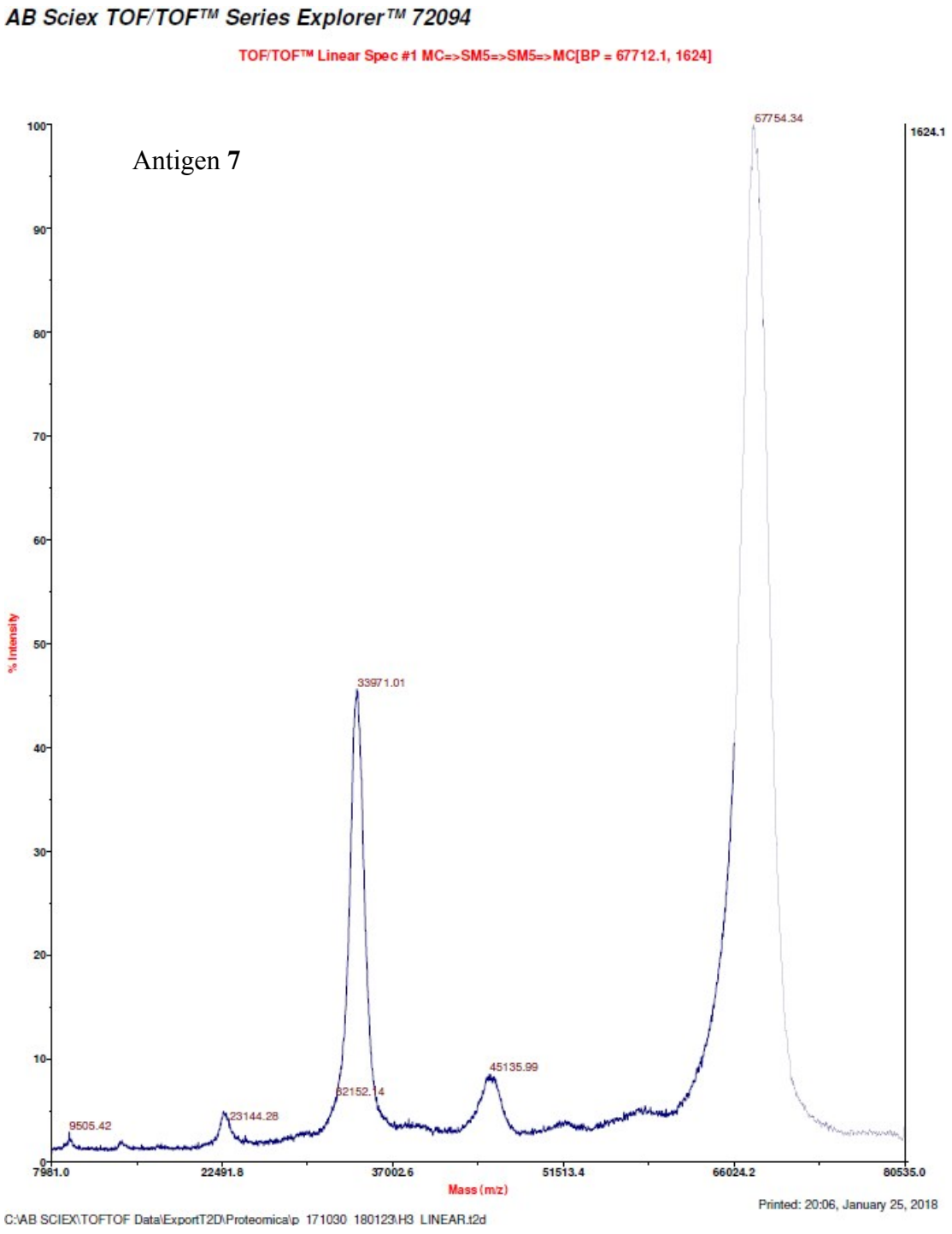
[MW(determinant)-MW(protein)]/MW(hapten).

The incremental change in molecular weight due to incorporation of hapten molecules to protein corresponds to the number of hapten molecules per protein molecule.

Control HSA



279 PG antigens



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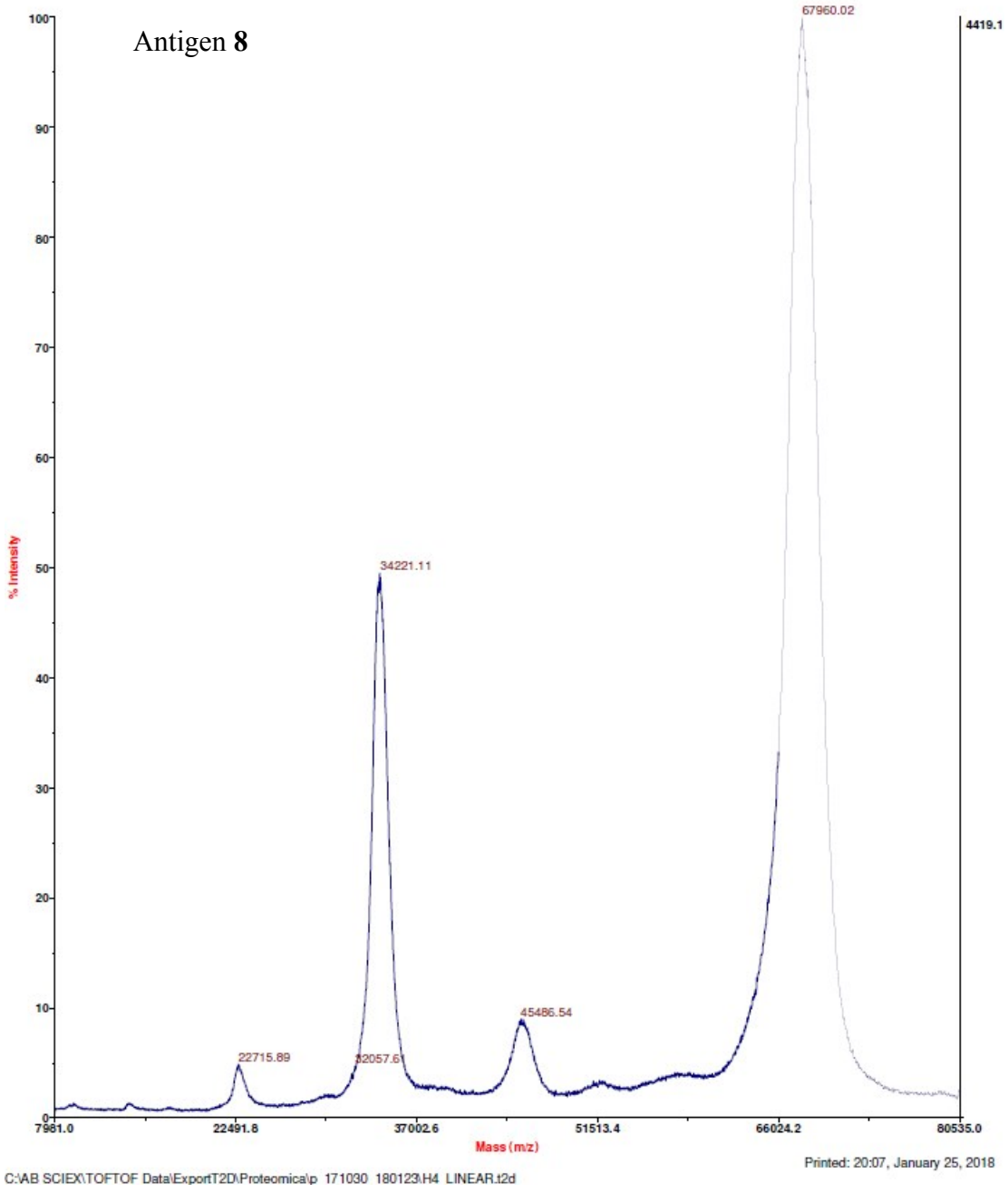
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TOF/TOF™ Linear Spec #1 MC=>SM5=>MC[BP = 67894.4, 4419]
Antigen 8
67960.02
4419.1
% Intensity
34221.11
45486.54
22715.89
32057.61
7981.0
22491.8
37002.6
51513.4
66024.2
80535.0
Mass (m/z)
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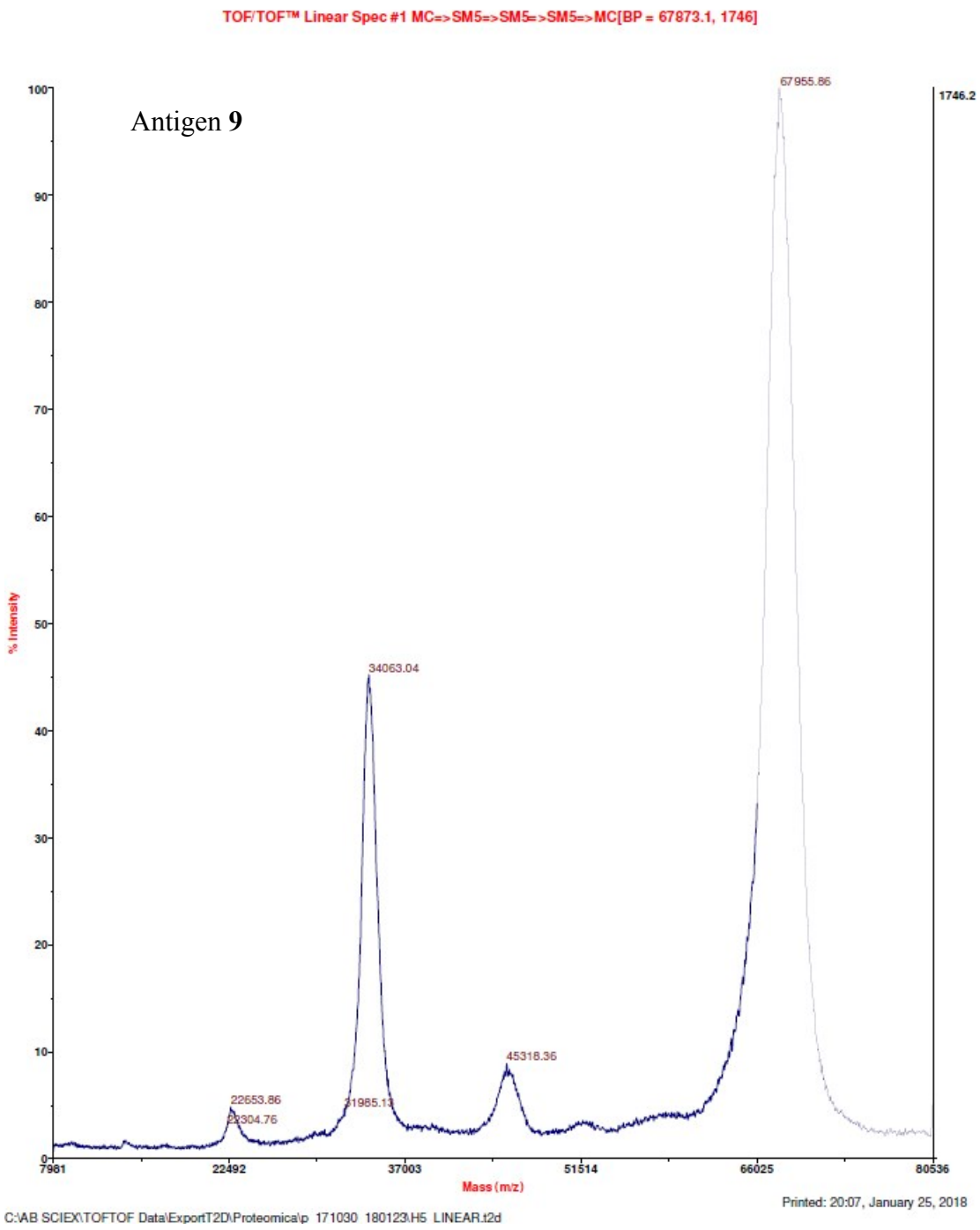
281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TOF/TOF™ Linear Spec #1 MC=>SM5=>SM5=>SM5=>MC[BP = 67873.1, 1746]
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1746.2
% Intensity
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45318.36
22653.86
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31985.13
Mass (m/z)
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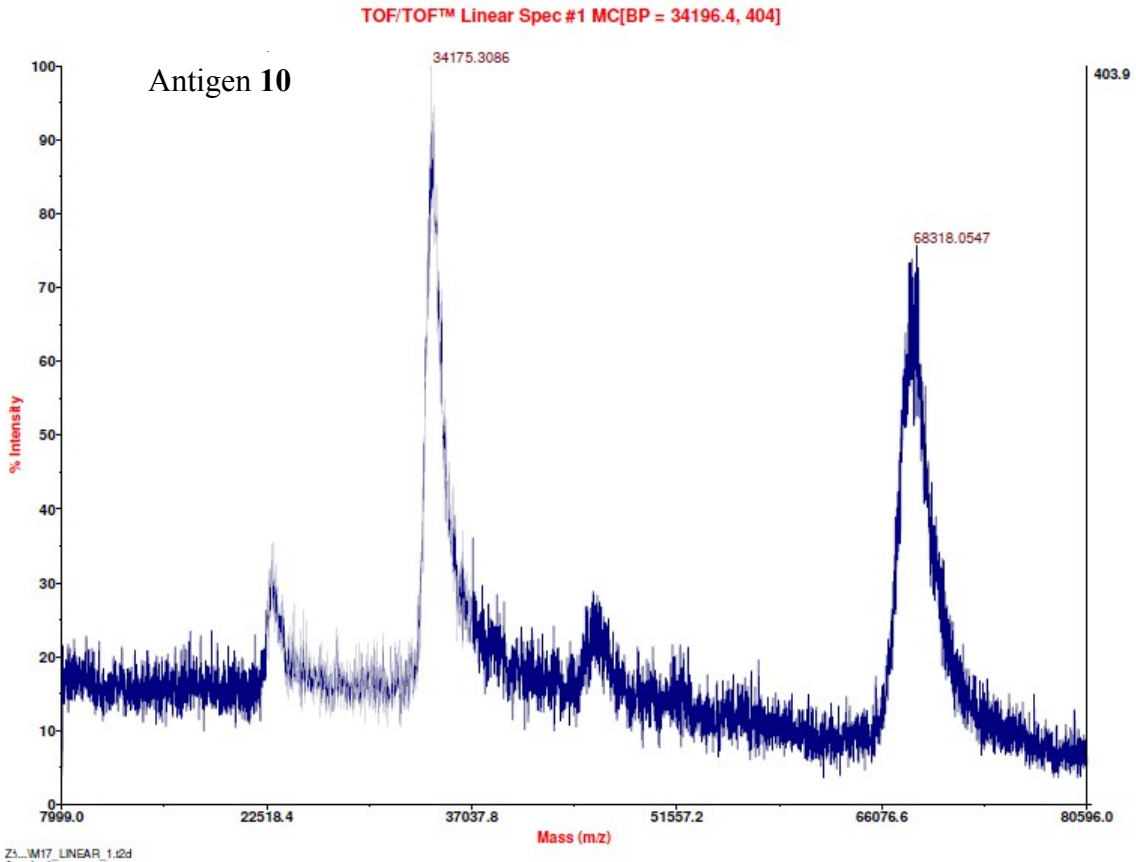
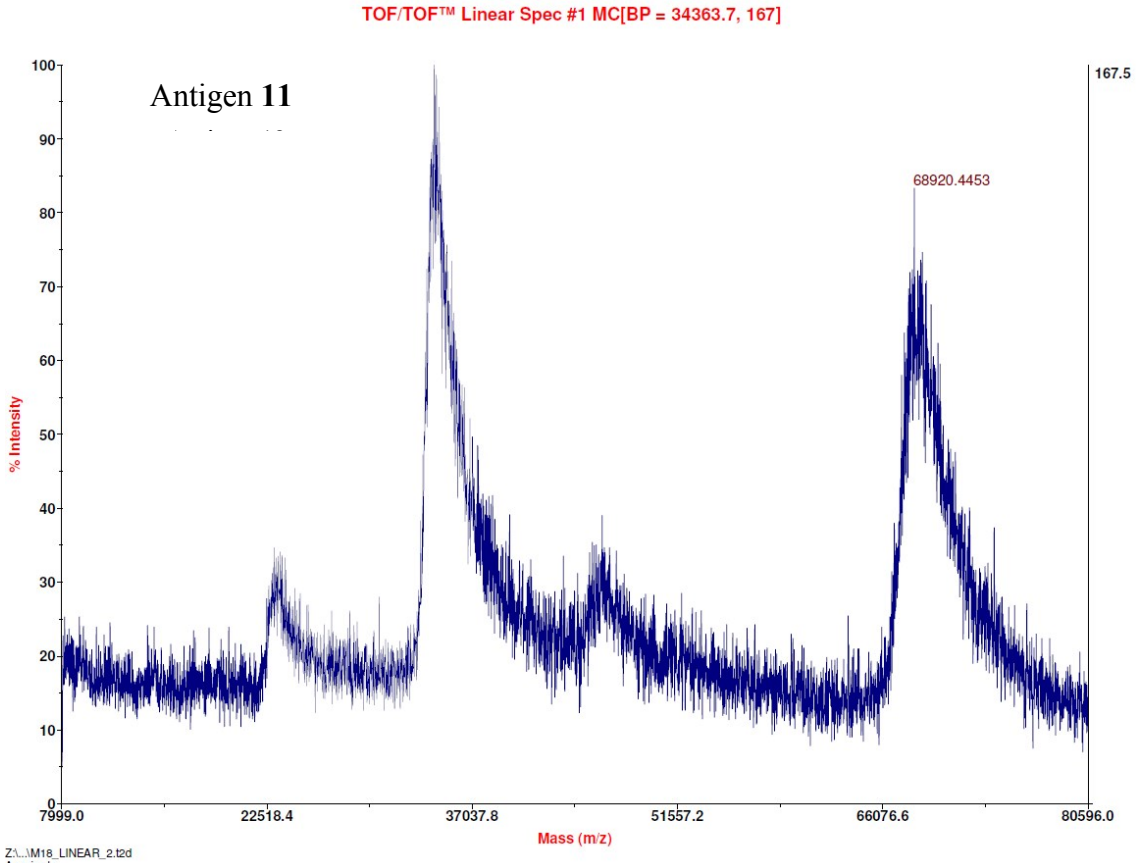
282

AMX antigens





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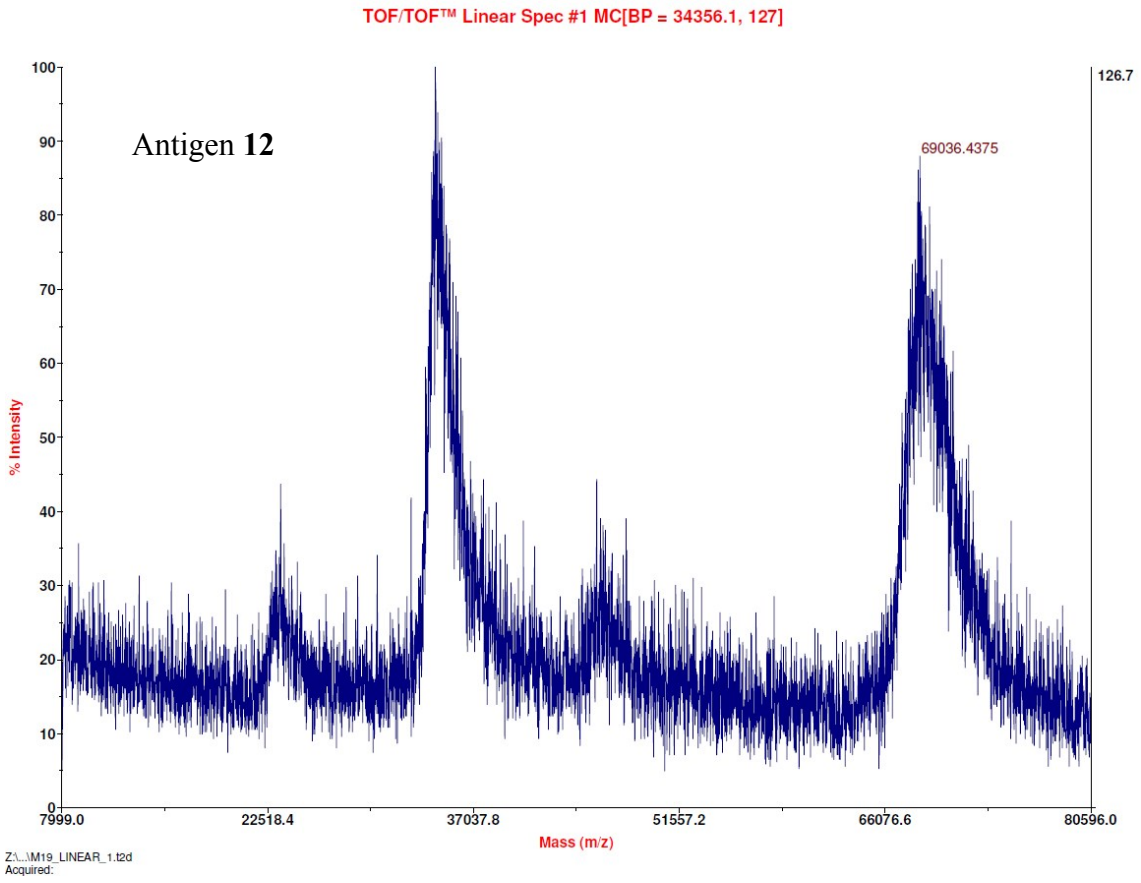
287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Antigen **12**
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126.7
% Intensity
Mass (m/z)
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22518.4
37037.8
51557.2
66076.6
80596.0
Z:\...\M19_LINEAR_1.t2d
Acquired:

7. Assay protocol for the evaluation of the structural antigens

Assays consist on the detection of specific IgG (Figure S1, assay I) and IgE (Figure S1, assays II and III) on standard DVDs (CD Rohling-up GmbH, Saarbrücken, Germany). For that, antigens (40 µg/mL) and controls (negative and positive), prepared in printing buffer, were spotted in microarray format (20 arrays per disk of 4 × 4 spots, Figure S2a) by dispensing 25 nL of each one, using a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA). The spots were 500 µm in diameter with a center-to-center distance of 1.0 mm. Within each microarray (Figure S2b-c), spots for each antigen (two replicates, position 3-8) and negative (HSA, position 1) and positive (rabbit IgG or human IgE, position 2) controls are included. After printing, the DVD was incubated for 16 h at 37 ºC.

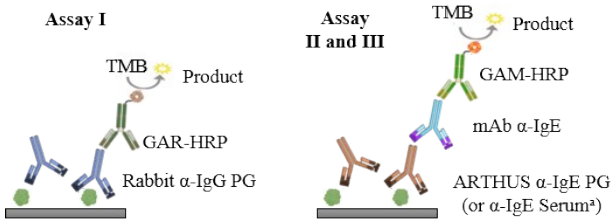

Figure S1: Scheme of the microimmunoassays I, II and III based on a direct format with colorimetric detection. [a]α-IgE serum from allergic patients and controls was used in assay III.

Figure S2: A) Image of the array on the DVD surface; B) Lay-out of the antigens and controls printed on the DVD (Position 1: HSA, negative control, C(-); 2: human IgE, positive control, C(+); 3: Aztreonam–oyl antigen, negative control C(-); 4-7: antigen **1-4**; 8: Reference antigen); C) Representative image of the array with specific antigens and controls printed on the DVD. Artificial human serum specific to PG was used.

For the detection of specific IgG to penicillins, different dilutions (1/1,000-1/32,000) of rabbit sera and control (PBS-T) (25 µL per sample) were added to each array and incubated for 15 min. Then, the DVD was washed with PBS-T and water and 25 µL of the polyclonal secondary antibody GAR-HRP in PBS-T buffer (dilution 1/400) was added for 15 min followed by the washing step. For the detection of specific IgE, 25 µL of sample was added to each array. Samples were incubated for 15 min when artificial human serum[4] was used, while serum samples from allergic patients and controls were incubated for 30 min. After washing, 25 µL of mAb-IgE in PBS-T buffer (1.0 µg/mL) was added and incubated for 15 min. After washing as before, 25 µL of a 1/100 dilution of GAM-HRP was added for 15 min. Finally, immunoreaction was developed in all immunoassays by homogenously dispensing 1.0 mL of TMB along the whole disc surface. The reaction was stopped by washing the disk

with water after 15 min. Signals were read by a modified DVD drive and the data was analysed as previously described [5,6]. All experiments were repeated 3 times.

BLC-specific IgE levels expressed as units of specific IgE (IU/mL) were determined, using the 3rd WHO standard for total serum IgE content involving heterologous interpolation. The calibration curve was built performing a sandwich immunoassay where 3rd WHO International Standard was used as calibrator and Omalizumab[7] as the capture antibody. All the other immunoreagents were the same as used for the determination of specific IgE (assays II and III). The standard data points, signal versus semi-log concentration, were the mean of 10 curves performed on different disks during several days. A four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 11 software.

Concentrations of specific IgE were calculated from the calibration curve for total IgE. Statistical significances between the obtained IgE concentrations for the diamine-derived antigens and the reference were determined by the Holm-Sidak method using the SigmaPlot 11 software and P values of <0.001 were considered significant.

8. Patients

The study of the reactivity of the prepared antigens towards the sera from allergic patients included patients (I) whose culprit drug was PG, AMX or augmentin and (II) whose culprit drug was another BLC. A cohort of 35 subjects with negative skin test to BLCs and with good tolerance to them were used as controls. Clinical characteristics from the 35 patients included in the study are shown in Supplementary Table S1. All samples from patients and controls were kindly provided by the Hospital Universitari i Politènic La Fe, Valencia, Spain, and informed consent for the diagnostic procedures was obtained from all patients. Patients were diagnosed following the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, in vitro tests or drug provocation test, when necessary. This study was approved by the Hospital Universitari i Politènic La Fe ethical review committee. All experiments were performed in accordance with the relevant guidelines and regulations.

Table S1: Clinical characteristics of the allergic patients.

Patient	Sex[a]	Year birth	Clinical manifestation	Clinical Entity	Time
01	M	1969	Augmentin	Anaphylaxis	Immediate
02	F	1978	Augmentin	Anaphylaxis	Immediate
03	M	1936	Augmentin	Anaphylaxis	Immediate
04	F	1939	Cefazolin	Anaphylaxis	Immediate
05	M	1949	Augmentin	Cutaneous	Delayed
06	F	1948	Augmentin	Anaphylaxis	Immediate
07	F	1967	Amoxicillin	Anaphylaxis	Immediate
08	F	1960	Augmentin	Anaphylaxis	Immediate
09	M	1956	Augmentin	Cutaneous	Delayed
10	F	1982	Cefuroxime	Anaphylaxis	Immediate
11	M	1972	Augmentin	Cutaneous	Delayed
12	F	1964	Augmentin	Cutaneous	Immediate
13	M	1978	Amoxicillin	Cutaneous	NR[b]
14	F	1961	Augmentin	Cutaneous	Immediate
15	F	1948	Amoxicillin	Inespecific	Immediate
16	F	1946	Penicillin G	Inespecific	Delayed
17	M	1977	Penicillin G	Cutaneous	Delayed
18	M	1953	Augmentin	Cutaneous	Immediate
19	M	1951	Amoxicillin	Cutaneous	Immediate
20	M	1964	Augmentin	Anaphylaxis	Immediate
21	M	1976	Amoxicillin	Cutaneous	Immediate
22	M	1961	Amoxicillin	Anaphylaxis	Immediate
23	M	1968	Amoxicillin	Cutaneous	NR
24	M	1965	Augmentin	Anaphylaxis	Delayed
25	M	1982	Amoxicillin	Cutaneous	Delayed
26	M	1982	Amoxicillin	Anaphylaxis	Immediate
27	F	1970	Piperacillin	Cutaneous	Immediate
28	F	1951	Amoxicillin	Cutaneous	Immediate
29	F	1982	Augmentin	Cutaneous	Immediate
30	F	1984	Augmentin	Cutaneous	Delayed
31	F	1963	Augmentin	Cutaneous	NR
32	F	1972	Augmentin	Anaphylaxis	Immediate
33	F	1962	Cefuroxime	Cutaneous	Delayed
34	M	1963	Amoxicillin	Cutaneous	Immediate
35	M	1975	Augmentin	Anaphylaxis	NR

[a]Sex: F=Female, M=Male; [b]NR=No reported in the clinical history

Table S2: Results of the analysis of human serum samples

	Prick-test[a]		ImmunoCAP (in IU/mL[b])		Multiplex Immunoassay[c] (in IU/mL)			
					Antigen No			
					PG		AMX	
Patient No	PG	AMX	PG	AMX	1b	3b	4b	5b
01	N	N	13.60	10.20	0.23	0.48	<LOD[e]	<LOD
02	N	P	0.03	0.16	0.34	0.23	0.10	0.09
03	N	P	2.16	1.64	3.65	0.20	0.07	<LOD
04	N	P	0.00	0.82	<LOD	0.44	<LOD	<LOD
05	NR	NR	2.14	1.01	0.66	0.18	0.07	<LOD
06	N	P	1.83	0.91	3.47	<LOD	<LOD	<LOD
07	NR	NR	1.02	0.79	1.11	0.21	0.10	0.08
08	N	P	0.08	0.13	0.15	0.16	<LOD	<LOD
09	N	N	0.00	0.95	<LOD	2.75	<LOD	<LOD
10	N	P	0.09	0.74	<LOD	<LOD	<LOD	<LOD
11	N	P	0.01	0.03	<LOD	<LOD		
12	N	P	0.02	0.04	<LOD	0.20		
13	N	P	0.00	0.15	0.11	<LOD		
14	N	P	0.12	0.29	<LOD	<LOD	<LOD	0.28
15	N	N	0.03	0.07	0.18	<LOD		
16	N	N	0.01	0.03	0.16	<LOD		
17	N	N	0.04	0.06	<LOD	<LOD		
18	N	P	0.30	0.24	<LOD	<LOD	<LOD	<LOD
19	NR	N	0.02	0.12	0.16	<LOD		
20	P	NR	0.03	0.08	0.14	0.21		
21	N	P	0.17	0.46	0.08	<LOD		
22	P	P	0.00	0.03	<LOD	<LOD		
23	P	P	0.00	0.09	<LOD	<LOD		
24	N	P	0.17	0.33	0.21	<LOD		
25	N	P	0.03	0.24	0.20	<LOD	<LOD	<LOD
26	N	N	0.43	0.99	0.62	<LOD	<LOD	<LOD
27	N	N	0.00	0.00	0.46	<LOD		
28	N	P	0.00	0.00	0.25	<LOD		
29	NR	NR	3.01	7.02	2.62	1.39	0.23	0.40
30	N	N	0.05	0.41	<LOD	<LOD	<LOD	<LOD
31	N	P	0.00	0.01	0.15	<LOD		
32	P	P	0.03	0.24	<LOD	<LOD		
33	N	N	0.00	0.01	0.49	<LOD		
34	N	P	0.01	0.69	<LOD	<LOD	<LOD	<LOD
35	N	P	0.08	0.09	<LOD	<LOD	<LOD	0.07

Immunoassay values are the mean of at least three replicates and relative standard deviation (RSD) ranged from 4 to 13%. [a] P= Positive, N= Negative, NR=Not Realized; [b] IU/mL= 2.4 ng/mL; [c]: Multiplex-DVD assay, using H1–oyl antigens; [d] Augmentin is a

356 combination of AMX and potassium clavulanate; [e] <LOD= Value below the limit of
357 detection (LOD).

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9. Experimental references

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