

Note

## The biofilm matrix of *Pseudomonas* sp. OX1 grown on phenol is mainly constituted by alginate oligosaccharides

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**Abstract**—The structure of the major constituent of the biofilm matrix produced by *Pseudomonas* sp. OX1, when grown on phenol as the sole carbon source is described. This investigation, carried out by chemical analysis, NMR spectroscopy and MALDI-TOF MS spectrometry, showed the presence of an oligosaccharide blend with the typical alginate structure, namely (1→4) substituted β-D-mannuronic (ManA) and α-L-guluronic acid (GulA). GulA residues were non-acetylated whereas ManA was always O-acetylated at C-2 or C-3.

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Microbial biofilm matrices are ubiquitous in aquatic and terrestrial ecosystems as well as on man-made materials. Surface-associated microbial activity and colonization, ubiquitously occurs through biofilm formation both in natural and artificial environments. Biofilms are dense bacterial communities characterized by close association of bacterial cells generally attached to a solid surface and surrounded by a saccharide matrix. Exopolysaccharides (EPSs) are the major components of bacterial biofilm matrix. They are secreted by microorganisms and play an essential role in biofilm structure and in microbial activity. They mediate the transport of chemicals to and from the microorganisms. Moreover, they also show ion exchange properties due to negatively charged surface functional groups, which bind to cationic species. EPS matrix also provides an effective barrier that restricts penetration of chemically reactive biocides, antibiotics and antimicrobial agents. It has been shown that bacteria living in biofilm matrix can be up to 1000 times more resistant to antibacterial com-

pounds than planktonic bacteria. This has been taken as an indication that the mechanisms involved in biofilm-mediated resistance to antimicrobials may differ from those responsible for antimicrobial resistance in planktonic bacteria.

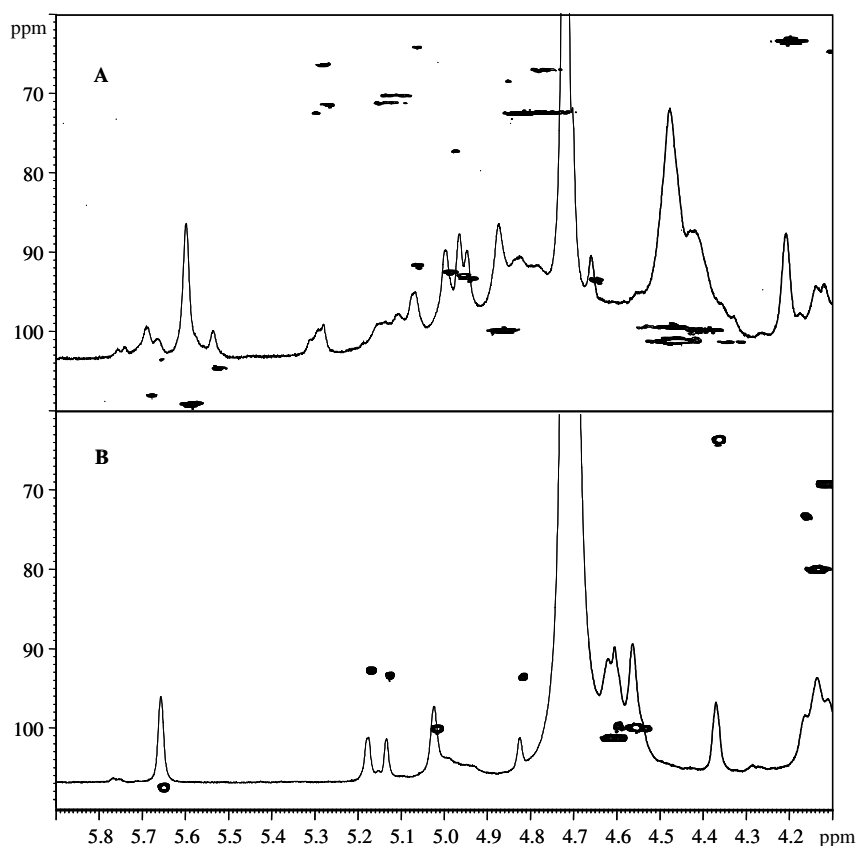
*Pseudomonas* sp. OX1 (formerly known as *Pseudomonas stutzeri* OX1)<sup>1</sup> is a Gram-negative bacterium, originally isolated from activated sludge of a wastewater treatment plant.<sup>2</sup> Its survival in such a contaminated habitat has been attributed to its ability to use aromatic compounds like phenol, dimethylphenol, cresol and *o*-xylene, as a source of carbon and energy. These molecules are converted by a complex series of enzymatic systems<sup>3–5</sup> into dihydroxylated derivatives, which are subsequently transformed into citric acid cycle<sup>5</sup> intermediates. To date, some of these enzymatic systems have been purified and characterized, namely, toluene-*o*-xylene monooxygenase,<sup>3,4</sup> phenol hydroxylase,<sup>5</sup> and catechol 2,3 dioxxygenase. It has been hypothesized<sup>6</sup> that, besides these specific metabolic pathways, microorganisms like *Pseudomonas* sp. OX1 must have developed other biochemical features for their survival under harsh conditions such as the presence of organic solvents at high concentrations. Previous studies have already

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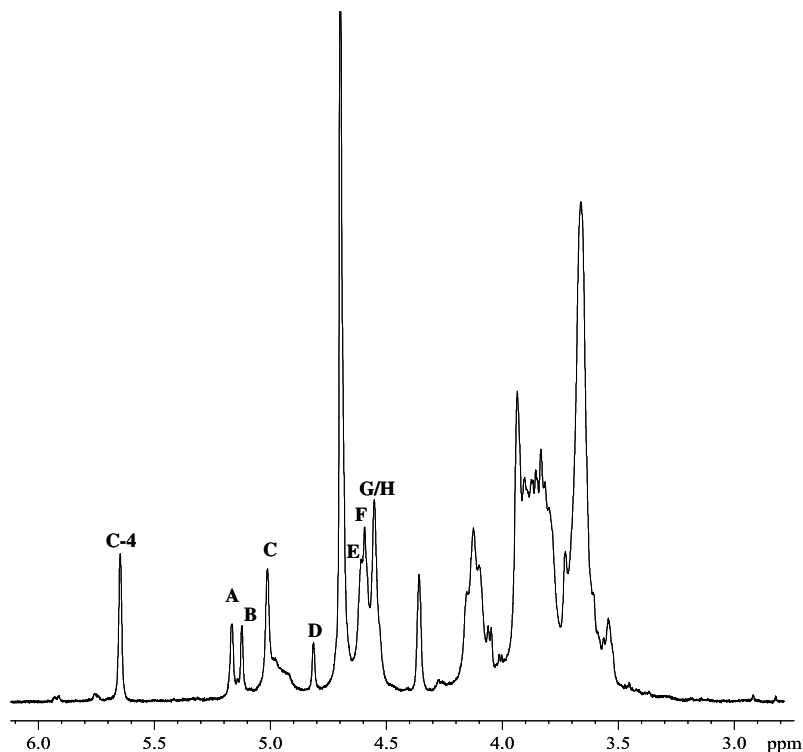
shown that the outer membrane of Gram-negative bacteria is involved in adaptation mechanisms to hostile habitats,<sup>6</sup> and that changes occur in inner and outer membranes,<sup>7</sup> for example, fatty acids and phospholipids composition, under different growth conditions. In particular, we have already examined the role of lipopolysaccharides in these accommodation mechanisms.<sup>8–10</sup> Recently, we have started the chemical study of the biofilm matrix produced by *Pseudomonas* sp. OX1 grown under stress conditions. In this paper we describe the structure of the major constituent of the biofilm matrix produced by the microorganism when grown on phenol as the sole carbon source. This investigation, carried out by chemical analysis, NMR spectroscopy and MALDI-TOF MS spectrometry, showed the presence of an oligosaccharide blend with alginate structure, (1→4) substituted  $\beta$ -D-mannuronic (ManA) and  $\alpha$ -L-guluronic acid (GulA) residues.

The oligosaccharide fraction was isolated from the supernatant of *Pseudomonas* sp. OX1 cultures grown on phenol and was purified by GPC and HPLC. The chemical analysis carried out by GLC of the acetylated *O*-methyl and *O*-oct-2-yl glycosides revealed the presence of GulA and ManA in L and D configurations, respectively. The  $^1\text{H}$  NMR spectrum showed a complex

saccharide fraction (Fig. 1A) with highly crowded anomeric and ring proton regions and, at high field, several methyl signals of acetyl groups. The HSQC spectrum (Fig. 1A) indicated that most of the signals in the anomeric region were correlated to carbon signals in the region 67–77 ppm, thus indicating that these were due to protons geminal to *O*-acetyl group. In order to simplify the analysis, an aliquot of the sample was *O*-deacetylated with ammonium hydroxide at room temperature for 16 h. The  $^1\text{H}$  NMR spectrum recorded after work-up of the reaction, showed a saccharide mixture that was much reduced in its heterogeneity (Figs. 1B and 2). A complete set of 2D NMR experiments (DQF-COSY, TOCSY, ROESY,  $^{13}\text{C}$ - $^1\text{H}$  HSQC,  $^{13}\text{C}$ - $^1\text{H}$  HMBC) was carried out at 600 MHz with a cryogenic probe. The anomeric region of the  $^1\text{H}$  NMR spectrum showed several signals (Fig. 1B and Table 1) all belonging to ManA and GulA units in different chemical/magnetic environments. In particular, numerous signals were present in the region of anomeric signals of  $\beta$ -ManA, and chemical shift assignment was performed for the most relevant spin systems. Identification of the residues was achieved by the complete assignment of all signals and by determination of the  $^3J_{\text{H,H}}$  coupling constants. All spin systems were recognized



**Figure 1.** Zoom of the anomeric region of the  $^1\text{H}$ ,  $^{13}\text{C}$ -HSQC and, overlapped, of  $^1\text{H}$  NMR spectra of oligosaccharide fraction isolated from the supernatant of *Pseudomonas* sp. OX1 cultures before (A) and after (B) mild de-*O*-acetylation. Spectra were recorded in  $\text{D}_2\text{O}$  at 25 °C.



**Figure 2.**  $^1\text{H}$  NMR spectrum of the de-O-acetylated oligosaccharides from the biofilm matrix of *Pseudomonas* sp. OX1.

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  (*italic*) NMR chemical shifts (ppm) for the main monosaccharides residues identified in the exopolysaccharides matrix of *Pseudomonas* sp. OX1

Residue	1	2	3	4	5	6
$\rightarrow$ 4)- $\alpha$ -GulA	5.164	3.728	3.902	4.128	4.104	—
A	<i>92.7</i>	<i>70.2</i>	<i>69.4</i>	<i>80.0</i>	<i>69.1</i>	<i>174.6</i>
$\rightarrow$ 4)- $\alpha$ -GulA	5.120	3.835	3.879	3.831	4.056	—
B	<i>93.4</i>	<i>70.2</i>	<i>69.0</i>	<i>78.3</i>	<i>72.7</i>	<i>174.2</i>
$\alpha$ - $\Delta$ GulA-(1 $\rightarrow$ )	5.016	3.856	4.359	5.643	—	—
C	<i>100.1</i>	<i>66.2</i>	<i>63.7</i>	<i>107.5</i>	<i>145.5</i>	<i>169.5</i>
$\rightarrow$ 4)- $\beta$ -GulA	4.812	3.807	4.130	3.905	4.15	—
D	<i>93.5</i>	<i>70.6</i>	<i>73.3</i>	<i>78.5</i>	<i>73.3</i>	<i>175.1</i>
$\rightarrow$ 4)- $\beta$ -ManA-(1-	4.610	3.831	n.d.	n.d.	n.d.	—
E	<i>101.4</i>	<i>69.9</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
$\rightarrow$ 4)- $\beta$ -ManA-(1 $\rightarrow$ )	4.590	3.941	3.660	3.798	3.650	—
F	<i>99.7</i>	<i>69.8</i>	<i>71.3</i>	<i>78.3</i>	<i>75.8</i>	<i>174.2</i>
$\rightarrow$ 4)- $\beta$ -ManA-(1 $\rightarrow$ )	4.551	3.929	3.657	3.828	3.671	—
G	<i>100.0</i>	<i>69.8</i>	<i>71.3</i>	<i>78.3</i>	<i>76.3</i>	<i>175.0</i>
$\rightarrow$ 4)- $\beta$ -ManA-(1 $\rightarrow$ )	4.526	3.880	3.555	3.828	3.670	—
H	<i>100.0</i>	<i>70.3</i>	<i>72.6</i>	<i>78.3</i>	<i>76.3</i>	<i>175.1</i>

Chemical shift values are measured at 300 K and refer to internal acetone ( $\delta_{\text{H}}$  2.225,  $\delta_{\text{C}}$  31.45).

as uronic acid units since, either in the COSY or in the TOCSY spectra, no H-6 proton signals were found, and in addition, in the HMBC spectrum the carboxy-group multitude of signals correlated to H-5 and H-4 proton signals of all spin systems (see below). Spin systems **A/B** and **D** (Fig. 2 and Table 1) were identified as different GulA residues present as  $\alpha$ - and  $\beta$ -reducing ends as confirmed by the shielded chemical shift value of their anomeric carbon signal and by comparison with the literature data.<sup>11</sup> Spin systems **E–H** were all attributed

to  $\beta$ -ManA residues, given the low  $^1J_{\text{C}-1,\text{H}-1}$  and  $^3J_{\text{H}-1,\text{H}-2}$  values, approximately 165 and 1.0 Hz. The  $\beta$ -anomeric orientation was further confirmed by intra-residual NOE effect between H-1 and H-3/H-5 signals found in the ROESY spectrum. Spin system **C** was identified as a 4-deoxy- $\alpha$ -L-erythro-hex-4-enopyranosyl-uronic acid ( $\Delta$ HexA) residue. Actually, in the TOCSY and COSY spectra, correlations within **C** spin system ended at H-4 at low fields (5.643 ppm) that was in turn correlated, in the  $^1\text{H}$ ,  $^{13}\text{C}$ -HSQC spectrum, to an olefin

carbon signal at 107.5 ppm. This residue clearly derived from an uronic acid residue that lost the substituent at O-4 by a  $\beta$ -elimination, recognizable either as a former  $\alpha$ -GulA residue or a  $\beta$ -ManA, since the loss of chirality at C-5 for both monosaccharides leads to the same product. All the ManA residues and the reducing end guluronic residue were found to be O-4 substituted, as suggested by the low field displacement of their C-4 NMR signals (all around 80 ppm, Table 1). This information was confirmed by the HMBC spectrum that showed inter-residue scalar correlations H-1/C-4 and C-1/H-4 of two vicinal residues.

The MALDI MS spectrum (Fig. 3) of the O-deacetylated product confirmed the presence of an oligosaccharide blend. In fact, few ion peaks were visible and the most abundant ion at  $m/z$  1056.2 contained six residues, the unsaturated guluronic and the uronic acid residues. The other ion peaks in the spectrum differed for the presence or the absence of single uronic acid units. The higher molecular weight ion species was visible at  $m/z$  2111.9 and contained 12 residues, one of which was the unsaturated hexuronic acid residue.

The acetyl groups were assigned by 2D NMR analysis of the native oligosaccharide mixture that showed that the chemical shift of guluronate residues was in agreement with those of a non-substituted residue whereas H-2 and H-3 chemical shifts of ManA spin systems were alternatively shifted to lower field because of acetylation. In fact, in the COSY spectrum when H-2 was shifted downfield by acetylation it always correlated, to an upfield H-3 signal and similarly, H-3 downfield signal always correlated to an upfield H-2.

Therefore, it can be concluded that GulA residues are always non-acetylated whereas ManA is always O-

acetylated at C-2 and C-3. Thus, from the data above, it was possible to identify the oligosaccharides of the biofilm matrix of *Pseudomonas* sp. OX1 as alginate, that is (1 $\rightarrow$ 4)-linked  $\beta$ -D-ManA and  $\alpha$ -L-guluronic acid residues.

Recent studies<sup>11</sup> have revealed that alginate hydrolysates and their derivatives exhibit many important bioactivities, such as stimulating human keratinocytes, accelerating plant root growth and enhancing penicillin production from cultures of *Penicillium chrysogenum*.

In this present case, the alginate oligosaccharides were all constituted by a mannan chain starting with a guluronate residue, whereas the monosaccharide at the non-reducing end was transformed in a hex-en-uronic residue, most likely by the action of an alginate lyase. A number of alginate lyases from brown algae, molluscs and bacteria have been described<sup>12</sup> and they usually cut alginate molecules by  $\beta$ -elimination, a mechanism similar to that of alkaline degradation to glycuronans. From the structure determined in the present study for *Pseudomonas* sp. OX1 it can be suggested that the lyase selectively hydrolyzes the glycoside linkage of GulA, since the oligosaccharides found always bear this monosaccharide at the reducing end. The GulA residues present in the alginate molecule derive by a postpolymerization epimerization at C-5 of ManA residues. The content and distribution of GulA residues strongly affects the physicochemical properties of alginate molecules. Therefore, the epimerization process is of great importance from an applied point of view. In all *Pseudomonas*, the epimerization process and consequently the content of GulA residues is biologically controlled by acetylation. In fact, the acetylated ManA residues cannot be converted to GulA since they are not recognized by the

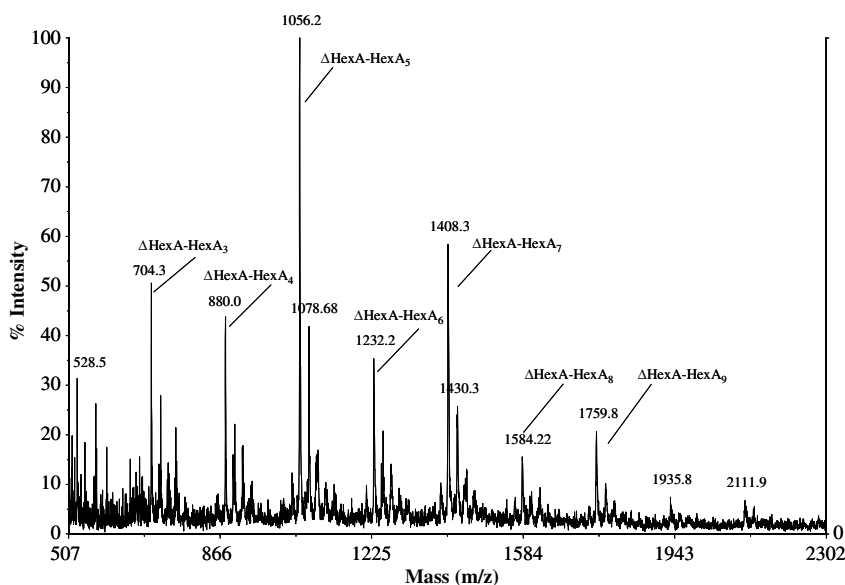


Figure 3. MALDI mass spectrum of the O-deacetylated oligosaccharides from the biofilm matrix of *Pseudomonas* sp. OX1.

mannuronate epimerase. Moreover, the high degree of acetylation is associated in alginates from *Pseudomonas* to an enhanced water-binding capacity that is important in particular dehydrating conditions, for example, for *P. aeruginosa* during infection and colonization of the lungs.<sup>13</sup> The hypothesis can be advanced such that the high degree of acetylation we have found in alginates from *Pseudomonas* is associated to an enhanced water-binding capacity that might be relevant under dehydrating conditions like those occurring during the growth of *Pseudomonas* sp. OX1 in the presence of aromatic hydrocarbons.

The presence of a high number of negative charges on the surface molecules (LPS and EPS) is another significant feature that plays an important role in hydrocarbons tolerance in *Pseudomonas* sp. OX1. In fact, the LPS of the bacterium grown on phenol contains a high number of phosphate and pyruvic acid residues<sup>8,9</sup> and, as it is demonstrated in this work, the biofilm matrix consists of highly charged oligosaccharides. This is in line with the very recent finding that *Rhodococcus* sp. 33, a benzene tolerant microorganism, produces a pyruvylated EPS and that the pyruvic acid moiety plays a crucial role in the tolerance and growth of the bacterium on benzene. In fact, *Rhodococcus* benzene sensitive mutants that did not produce EPS were able to grow on benzene, only after addition of wild type EPS, and were not able to grow after addition of depyruvylated EPS.<sup>14</sup>

## 1. Experimental

### 1.1. Growth of *Pseudomonas* sp. OX1

*Pseudomonas* sp. OX1 was routinely grown on M9-agar plates supplemented with 10 mM malic acid as the sole carbon source, at 27 °C. For growth in liquid medium, a single colony from a fresh plate was inoculated in 1 mL of the medium and grown overnight at 27 °C under constant shaking. This saturated culture was used to inoculate 100 mL of the same medium and grown at 27 °C up to about 1 OD<sub>600</sub>. Final growth was carried out in 4 mM phenol as the sole carbon and energy source and was started by inoculating the appropriate volume of the latter culture into 1 L of fresh medium to 0.02 OD<sub>600</sub>. Cells were grown at 27 °C, until OD<sub>600</sub> = 1 and harvested by centrifugation at 3000g, for 15 min, at 4 °C.

### 1.2. Isolation of alginate oligosaccharides

The supernatant (1 L) recovered after cell removal was reduced by vacuum evaporation, desalted by gel-permeation chromatography on a Sephadex G-10 column eluted with 50 mM NH<sub>4</sub>HCO<sub>3</sub> buffer and freeze-dried,

obtaining 77 mg of dry mass. Crude extract was purified by gel-permeation chromatography on a Sephacryl S-400 column eluted with 50 mM NH<sub>4</sub>HCO<sub>3</sub> buffer. The carbohydrate containing fraction (65 mg) was further purified by size exclusion HPLC using a TSK G-5000 PW<sub>XL</sub> analytical grade (Tosoh, Japan) column in water, obtaining the pure alginate fraction. O-Deacetylation was achieved by treating the alginate fraction with 15% aqueous ammonia at 25 °C for 16 h. Determination of monosaccharides and their absolute configuration GLC and GLC-MS were all carried out as described elsewhere.<sup>8,9,15</sup>

### 1.3. NMR spectroscopy

1D and 2D <sup>1</sup>H NMR spectra were recorded on a solution of 3 mg in 0.6 mL of D<sub>2</sub>O, at 25 °C. <sup>1</sup>H and <sup>13</sup>C NMR experiments were carried out using a Bruker DRX-600 equipped with a cryogenic probe. Spectra were calibrated with internal acetone δ<sub>H</sub> 2.225, δ<sub>C</sub> 31.45. Rotating frame Overhauser enhancement spectroscopy (ROESY) was measured using data sets (*t*<sub>1</sub> · *t*<sub>2</sub>) of 4096 · 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 · 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 120 ms, using data sets (*t*<sub>1</sub> · *t*<sub>2</sub>) of 4096 · 512 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 · 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the <sup>1</sup>H-detected mode with proton decoupling in the <sup>13</sup>C domain, using data sets of 2048 · 512 points, and 100 scans were acquired for each *t*<sub>1</sub> value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.<sup>16</sup> A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048 · 1024 points using forward linear prediction extrapolation.

### 1.4. Mass spectrometry

MALDI-TOF MS of the O-deacetylated fraction was performed with a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA), in the negative-ion mode at an acceleration voltage of 24 kV. The sample was dissolved in water at a concentration of 1 µg/µL. One microlitre of the sample was then mixed (directly on the metallic sample surface)

with 1  $\mu$ L of a 20 mg/mL solution of 2,5-dihydroxybenzoic acid (Aldrich, Steinheim, Germany) in acetonitrile/0.1 M trifluoroacetic acid 7:3. The mass spectra are the result of approximately 150 scans.

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