Electrospray ionization mass spectrometry analysis of polyisoprenoid alcohols via Li$^+$ cationization

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Abstract

Direct analysis of polyisoprenoids by electrospray ionization mass spectrometry (ESI–MS) often produces poor results requiring off-line time and sample-consuming derivatization techniques. We describe a simple ESI–MS approach for the direct analysis of polyisoprenoids using several dolichols and polyprenols with different chain sizes as proof-of-principle cases. Lithium iodide is used to promote cationization by intense formation of [M + Li]$^+$ adducts. Thus, detection of polyisoprenoids with mass determination can be performed with high sensitivity (limit of detection [LOD] $\approx 100$ pm), whereas characteristic collision-induced dissociations observed for both dolichols and polyprenols permit investigation of their structure. Using ESI(Li$^+$)–MS and ESI(Li$^+$)–MS/MS analysis, we screened for polyprenol products of an octaprenyl pyrophosphate synthase of Plasmodium falciparum and dolichols in a complex mixture of compounds produced by Leishmania amazonensis and P. falciparum.

Keywords: Mass spectrometry; Polyisoprenoids; Polyprenols; Dolichols; Plasmodium falciparum; Leishmania amazonensis; Lithium

Isoprenoids, which are composed of a common five-carbon unit polymer backbone structure [1], are the most numerous and diverse group of natural products, covering more than 30,000 different compounds [2]. Many isoprenoids are linear polymers named polyisoprenoid alcohols, which are divided into two main groups: (i) dolichols (Scheme 1A), which are $\alpha$-saturated isoprenoid alcohols found in all animal cells and some bacteria, parasites, fungi, and plants, and (ii) polyprenols (Scheme 1B), which are $\alpha$-unsaturated isoprenoid alcohols found in the green tissues of many plants, bacteria, yeast, and parasites.

Animals, bacteria, and some parasites such as Leishmania amazonensis are known to biosynthesize isoprenoids via the mevalonic acid pathway [3,4], whereas plants and some bacteria and parasites such as Plasmodium falciparum use the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [5,6]. These two pathways have been found to occur concurrently in organisms such as bacteria and fungi [7].

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Polyprenols and dolichols are found in cells as free alcohols and esters [8]. Phosphorylated polyprenoids have also been found and play a role as cofactors in the biosynthesis of glycoproteins and glycosylphosphatidyl inositol (GPI) anchors or bacteria peptidoglycans [9,10]. They have also been postulated to serve as donors of isoprenoid (GPI) anchors or bacteria peptidoglycans [9,10]. They have also been found and play a role as cofactors in the biosynthesis of glycoproteins and glycosylphosphatidyl inositol (GPI) anchors or bacteria peptidoglycans [9,10].

The main biological role of free polyprenoid alcohols and carboxylic esters probably is related to their ability to increase the permeability and fluidity of cell membranes [12] and their involvement in the transport of vacuolar proteins as well as in the transport mechanisms involving the endoplasmic reticulum [13]. Dolichols can also participate in protein isoprenylation [11], a posttranslational modification of proteins involved in events such as tumor cell growth and differentiation and cellular signaling [14,15].

The role of polyprenol-like substances in the biosynthesis of glycoconjugates has been studied extensively, and many mass spectrometric methods are used for analysis of polyprenoids in biological samples [16].

In electron ionization mass spectrometry (EI–MS) analysis [17,18], underivatized polyprenoids ionize efficiently and the EI mass spectra contain a large amount of structural information, but this may be masked by the presence of impurity peaks, especially in biological samples. EI-induced dissociation occurs extensively, forming numerous fragment ions, and makes the mass spectra difficult to interpret in terms of the structural characterization and identification of the molecular ion.

For analysis of polyprenoids with EI–MS, off-line derivatization to tert-butyl-dimethylsilyl (TBDMS) ethers [17] normally is used. The hydroxyl groups of polyprenoids are commonly converted to TBDMS ethers, improving sensitivity and the quality of structural information from EI–MS.

With soft ionization techniques such as fast atom bombardment mass spectrometry (FAB–MS) [17,19,20] and field desorption mass spectrometry (FD–MS) [21], analysis of underivatized polyprenoid compounds is also difficult. These molecules fail to ionize efficiently, and the mass spectra present poor structural information with a lack of molecular ions, and the extensive fragments observed often are not structurally characteristic. Off-line derivatization in sulfates [17] or phosphates [19] is necessary before MS analysis to increase polarity and facilitate protonation or deprotonation of the molecules.

Due to the high hydrophobicity of the polyprenoid alcohols [22], the same difficulties are found in electrospray ionization mass spectrometry (ESI–MS) as in other soft ionization techniques (FAB–MS and FD–MS) [17].

Although good-quality spectra normally are obtained after derivatization, with easy detection of molecular ions and fragment ions that reflect structural aspects, these preliminary off-line steps involving sample manipulation are time- and sample-consuming. We considered performing the ESI–MS analysis of polyprenoids in samples of parasites such as Plasmodium and Leishmania, for which very small amounts normally are employed, but realized that the use of derivatization would make this approach unviable.

More recently, efficient ionization of low-polar compounds such as lipids and steroids has been achieved with prior derivatization as their Na+ and Li+ adducts via the addition of sodium or lithium salts [23–26].

We report here that ESI–MS of polyprenoid alcohols occurs efficiently via Li+ cationization, that is, via their [M + Li]+ adducts. Dolichols and polyprenols are ionized efficiently by ESI(Li+)–MS, whereas dissociation via ESI(Li+) tandem mass spectrometry (ESI(Li+)–MS/MS) reveals detailed structural information. Therefore, we applied ESI(Li+)–MS and ESI(Li+)–MS/MS to screen for, and investigate the structures of, dolichols found in L. amazonensis and P. falciparum and were also able to identify several polyprenoid products of an octaprenyl pyrophosphate synthase of P. falciparum.

**Materials and methods**

**Chemicals**

Albumax I was purchased from Gibco (Carlsbad, CA, USA). RPMI 1640 medium, D-sorbitol, saponin, lithium acetate, sodium iodide, sodium iodate and authentic standards of geraniol (~98%), geranylergeraniol (~85%), and undecaprenol were purchased from Sigma (St. Louis, MO, USA). Authentic standards of dolichols of 11, 19, and 24 isoprene units and polyprenols of 8, 9, 10, 11, and 12 isoprene units were kindly provided by Tadeusz Chojnacki (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). HPLC-grade methanol, propan-2-ol, and chloroform were purchased from J.T. Baker (Phillipsburg, NJ, USA); 1-butanol and n-pentane were purchased from Carlo Erba (Rodano, Italy); and hexane was purchased from Mallinckrodt (Phillipsburg, NJ, USA). Lithium iodide hydrate (~98.0%) was purchased from Fluka (Buchs, Switzerland).

**Parasite cultures**

**Plasmodium falciparum culture**

*Plasmodium falciparum* 3D7 clone (isolate NF54) was cultivated according to the method of Trager and Jensen.
[27] as modified by Kimura and co-workers [28]. The parasites were grown under a gas atmosphere consisting of 5.05% CO₂, 4.93% O₂, and 90.20% N₂. Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears.

Cultures (~20% parasitemia) initially were synchronized in young ring forms (1–10 h after red blood cell invasion) by two treatments with 5% (w/v) D-sorbitol solution in water [29] and were maintained in culture until the schizont stage was reached (30–35 h after reinvasion). The cultures were centrifuged at 2000 g, and the parasites were isolated from erythrocytes by treatment with 0.1% (w/v) saponin for 5 min, followed by three washes with phosphate-buffered saline (PBS) (0.007 M Na₂HPO₄, 0.01 M NaH₂PO₄, pH 7.4, 0.15 M NaCl) at 10,000 g for 10 min. In this study, we used only schizont forms.

**Leishmania amazonensis culture**

*Leishmania* promastigotes were grown in M199 medium supplemented with 10% fetal bovine serum, 100 μM adenine, 10 μg/ml of heme, 40 mM Hapes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.4), 50 units/ml of penicillin, and 50 μg/ml of streptomycin, as described previously [30]. The parasites were collected from the culture, washed three times with PBS at 10,000g for 10 min, and stored in nitrogen. The strain used was *L. amazonensis* MHOM/BR/1975/M2903.

**Instrumentation**

The HPLC system used was a Gilson HPLC 322 pump (Gilson, Villiers-le-Bel, France) and a gradient module connected to a 152 UV–Vis detector, an 831 temperature regulator, and an FC203B fraction collector (Gilson). The samples were introduced into the HPLC columns through an inject valve with a 500-μl loop using a 500-μl Hamilton syringe (Reno, NV, USA). UniPoint software (Gilson) was used as the operational and analytical system.

ESI(Li⁺)-MS and ESI(Li⁺)-MS/MS were performed with a Finnigan LC-Duo trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The samples were analyzed by direct infusion into the ESI source using a Harvard syringe pump (model 11, Harvard, Holliston, MA, USA) operating at a 5- to 10-μl/min flow rate (Scheme 2C) or through an inject valve with a 10-μl loop using an OmniFit N₁ pressure system (OmniFit, Cambridge, UK) as a solvent pump set at 10 psi with a flow rate of 10 μl/min (Scheme 2B).

Spectra were acquired using LCQ Tune software, and data processing was performed using Xcalibur and Qual Browser software (version 1.2, Thermo Finnigan). The computer was an x86-based 2-GHz Pentium IV PC with 512 MB RAM.

**Extraction and preparation of samples**

*Leishmania amazonensis samples*

To analyze dolichol from *L. amazonensis*, freeze-dried pellets of promastigote parasites (~1 × 10¹¹) were extracted three times with 1 ml of hexane. The pooled extracts were dried under a nitrogen stream and stored at −70°C.

The extracts were resuspended in 400 μl of methanol, filtered through a 0.45-μm nylon filter (Advantec MFS, Dublin, CA, USA), and initially analyzed on an Ultra- sphere ODS C18 Beckman column (250 mm × 4.6 mm × 5 μm particle size, Beckman Instruments, Fullerton, CA, USA). A gradient elution system was used, with methanol/water (9:1, v/v) as solvent A and hexane/propan-2-ol/methanol (1:1:2, v/v/v) as solvent B. A linear gradient from 5 to 100% B over a period of 25 min was run, and then 100% B was pumped through for an additional 5 min. The flow rate was 1.5 ml/min [31]. The UV detector was set at 210 nm, and fractions were collected at 0.5-min intervals.

To improve purification of the sample for subsequent MS analysis, samples with the same retention times as polyisoprenoid standards of 10, 11, and 12 isoprene units and
the dolichol standard of 11 isoprene units were collected, dried under a nitrogen stream, resuspended in 400 µl of methanol, and reanalyzed on a Phenomenex Luna C18 column (250 mm x 4.6 mm x 5 µm particle size, Phenomenex, Torrance, CA, USA).

A gradient elution system was used with methanol/propan-2-ol/water (12:8:1, v/v/v) as solvent A and hexane/propan-2-ol (1:1, v/v) as solvent B. A linear gradient from 0 to 70% B over a period of 40 min was run, and then 70% B was pumped through for an additional 5 min. The flow rate was 1.0 ml/min (method adapted from Ref. [31]). The UV detector was set at 210 nm, and samples were collected at 1-min intervals. The samples with the same retention times as the polyprenol standards of 10, 11, and 12 isoprene units and dolichol standard of 11 isoprene units were collected, dried under a nitrogen stream, and stored at −70 °C.

**Plasmodium falciparum samples**

To analyze dolichol from *P. falciparum*, freeze-dried pellets of schizont parasites (~2 × 10^12) were extracted three times with 1 ml of hexane. The hexane extracts were dried under a nitrogen stream and stored at −70 °C for subsequent HPLC analysis.

The polisoprenoid products analyzed in this study were obtained from in vitro enzymatic reaction of a purified version of a native *P. falciparum* octaprenyl pyrophosphate synthase (PiOPPs) and a truncated recombinant form of this enzyme (TPIOPPs) with the substrates [32].

After enzymatic reaction in 0.3 ml of HKM buffer (100 mM Hepes, pH 7.5, 50 mM KCl, 0.5 mM MgCl₂, 0.1% Triton X-100), 10 µM farnesyl pyrophosphate (FPP), 10 µM isopentenyl pyrophosphate (IPP), and a suitable amount of enzyme solution (recombinant or purified native PiOPPs) for 30 min [32], polyprenyl diphosphate products were extracted with 1 ml of n-butanol and the pooled extracts were treated with potato acid phosphatase enzyme (according to the method of Fujii et al. [33]) to convert the polyprenyl diphosphates into their corresponding polyprenols. An additional extraction (three times with 1 ml of n-pentane) was performed, and the pool was dried under a nitrogen stream and stored at −70 °C for subsequent HPLC analysis.

For HPLC analysis, n-pentane extracts from enzymatic reaction and hexane extracts from schizont parasites were resuspended in 400 µl of methanol, filtered through a 0.45-µm nylon filter (Advantec), and analyzed on a Phenomenex Luna C18 column (250 mm x 4.6 mm x 5 µm particle size). A gradient elution system was used, with methanol/water (9:1, v/v) as solvent A and hexane/propan-2-ol/methanol (1:1:2, v/v/v) as solvent B. A linear gradient from 5 to 100% B over a period of 25 min was run, and then 100% B was pumped through for an additional 5 min. The flow rate was 1.5 ml/min [31]. The UV detector was set at 210 nm, and fractions were collected at 0.5-min intervals.

To analyze the octaprenyl pyrophosphate synthase products of both enzymes (TPIOPPs and PiOPPs), samples with the same retention times as the polisoprenoid standards of 8, 9, and 11 isoprene units were collected, dried under a nitrogen stream, and stored at −70 °C.

To analyze dolichol, samples with the same retention times as the dolichol standard of 11 isoprene units and polyprenol standard of 12 isoprene units were collected, dried under a nitrogen stream, and stored at −70 °C.

In all of the analyses, samples were collected in 1.5-ml plastic tubes (Eppendorf, Hamburg, Germany).

**Mass spectrometry**

To optimize the MS analysis, authentic standards of geranylgeraniol and dolichols of 11 and 24 isoprene units (calibration standards) in chloroform/methanol (1:1, v/v) containing 2 mM of lithium iodide were continuously injected directly into the ESI source of the Finnigan LCD-Duo ion trap mass spectrometer using a Harvard syringe pump (model 11) operating at a 10-µl/min flow rate (Scheme 2). The parameters for each analysis were tuned with the auto-tune operation in the LCQ Tune-Plus software (version 1.2.2).

For all of the analyses, the samples were resuspended in 10 µl chloroform/methanol (1:1, v/v) containing 2 mM of lithium iodide and were loaded into the 10-µl loop of the mass spectrometer (Scheme 2). The solvent (chloroform/methanol [1:1, v/v] containing 2 mM of lithium iodide) was pumped continuously using the OmniFit pressure system (Scheme 2B).

All ESI(Li⁺)–MS spectra were acquired in the positive ion mode, with spray voltage, capillary voltage, and capillary temperature set at 4.52 kV, 17 V, and 250 °C, respectively. For ESI(Li⁺)–MS/MS and ESI(Li⁺)–MS³, relative collision energy of 40% (2 eV) was applied in all of the analyses and the sheath (N₂) and collision (He) gas pressure settings were 80 and 20 arbitrary units, respectively. No in-source dissociation was attempted.

ESI(Li⁺)–MS spectra were acquired in both full ion mode and selective ion monitoring (SIM) mode over the m/z ranges presented in Table 1. The smoothing filter and background subtraction were used for data processing. For quantization, the Quan Browser software (version 1.2) was used.

**Standard solutions**

**HPLC standards**

To determine the retention times of the polisoprenoids in the HPLC experiments, solutions with 1 mM of dolichol of 11 isoprene units and polyprenols of 8, 9, 11, and 12 isoprene units were prepared in methanol. Equal volumes of each stock solution (50 µl) were mixed together and injected into the HPLC columns for analysis.

**Calibration standards**

To calibrate the mass spectrometer, solutions with of 1 µM of geranylgeraniol and dolichols of 11 and 24
isoprene units were prepared in chloroform/methanol (1:1, v/v) containing 2 mM of lithium iodide.

Analysis standards
To perform the MS analysis of the polyisoprenoid standards, solutions of 1 μM of geraniol, geranylgeraniol, polyprenols of 8, 9, 10, 11, and 12 isoprene units, and dolichols of 11, 19, and 24 isoprene units were prepared in chloroform/methanol (1:1, v/v). The solutions of polyprenol of 11 isoprene units and dolichol of 11 isoprene units were diluted separately (1:10, v/v) in chloroform/methanol (1:1, v/v) with different concentrations of lithium iodide (0, 2, 4, 10, 15, and 20 mM) to determine the optimal concentration of lithium iodide for ionization of the isoprene molecules.

After establishing the optimal concentration of lithium iodide (2 mM), the analysis solutions of all polyisoprenoid standards were serially diluted with chloroform/methanol (1:1, v/v) and 2 mM of lithium iodide to give solutions with concentrations of 1 μM, 10 μM, 100 μM, 1 nM, 10 nM, 100 nM, and 1 μM to determine the detection limit for each compound.

Limits of detection
To calculate the limit of detection (LOD) for each polyisoprenoid, we used a liquid chromatography mass spectrometry (LC–MS) method. This method fails to promote chromatogram separation of polyisoprenoids but provides a chromatography peak for each polyisoprenoid and allows the LOD to be calculated. A Phenomenex Luna C18 column (250 mm × 1.0 mm × 5 μm particle size) was placed between the divert valve and the ESI–MS source of the mass spectrometer, and an isocratic flow of chloroform/methanol (1:1, v/v) was set up using the Gilson HPLC 322 pump set at 0.5 ml/min and connected to a splitter (10:1) to reduce the flow rate to 50 μl/min (Scheme 2A). Lithium iodide dissolved in chloroform/methanol (1:1, v/v) was introduced directly into the ESI–MS source postcolumn by a Harvard syringe pump (model 11, flow rate 1 μl/min) as auxiliary solvent (Scheme 2B). The final concentration of lithium iodide in the sample was 2 mM.

Table 1
Molecular formulas, molecular weights, masses of [M + Li]+ ion (Da), and m/z range used for MS acquisition to determine LODs for the standards used in this study

<table>
<thead>
<tr>
<th>Standard</th>
<th>Molecular formula</th>
<th>Formula weight</th>
<th>Singly charged lithium adduct ion mass</th>
<th>m/z range (full ion mode)</th>
<th>m/z range (selective ion monitoring)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geranylgeraniol</td>
<td>C_{20}H_{30}O</td>
<td>290.48</td>
<td>297.38</td>
<td>50–500</td>
<td>297 ± 3</td>
</tr>
<tr>
<td>Polypropenol 8</td>
<td>C_{20}H_{30}O</td>
<td>562.95</td>
<td>569.85</td>
<td>400–600</td>
<td>570 ± 3</td>
</tr>
<tr>
<td>Polypropenol 9</td>
<td>C_{20}H_{30}O</td>
<td>631.06</td>
<td>637.96</td>
<td>550–700</td>
<td>637 ± 3</td>
</tr>
<tr>
<td>Polypropenol 10</td>
<td>C_{20}H_{30}O</td>
<td>699.18</td>
<td>706.08</td>
<td>650–900</td>
<td>706 ± 3</td>
</tr>
<tr>
<td>Polypropenol 11</td>
<td>C_{20}H_{30}O</td>
<td>767.30</td>
<td>774.20</td>
<td>700–850</td>
<td>774 ± 3</td>
</tr>
<tr>
<td>Dolichol 11</td>
<td>C_{20}H_{30}O</td>
<td>769.31</td>
<td>776.21</td>
<td>700–850</td>
<td>776 ± 3</td>
</tr>
<tr>
<td>Polypropenol 12</td>
<td>C_{20}H_{30}O</td>
<td>835.42</td>
<td>842.32</td>
<td>650–900</td>
<td>842 ± 3</td>
</tr>
<tr>
<td>Dolichol 12</td>
<td>C_{20}H_{30}O</td>
<td>837.43</td>
<td>844.33</td>
<td>750–900</td>
<td>844 ± 3</td>
</tr>
<tr>
<td>Dolichol 19</td>
<td>C_{20}H_{30}O</td>
<td>1314.24</td>
<td>1321.14</td>
<td>700–1400</td>
<td>1321 ± 3</td>
</tr>
<tr>
<td>Dolichol 24</td>
<td>C_{32}H_{46}O</td>
<td>1654.51</td>
<td>1659.71</td>
<td>1500–1800</td>
<td>1660 ± 3</td>
</tr>
</tbody>
</table>

Table 2
Detection limit (3:1 S/N ratio, in nM) of polyisoprenoid standards in the different analytical modes

<table>
<thead>
<tr>
<th>Standard</th>
<th>Full ion mode</th>
<th>Selective ion monitoring</th>
<th>MS/MS</th>
<th>MS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geraniol</td>
<td>0.25a</td>
<td>0.10a</td>
<td>1.00b</td>
<td>—</td>
</tr>
<tr>
<td>Geranylgeraniol</td>
<td>0.20a</td>
<td>0.10a</td>
<td>1.00b</td>
<td>—</td>
</tr>
<tr>
<td>Dolichol 11</td>
<td>0.15a</td>
<td>0.10a</td>
<td>1.00b</td>
<td>—</td>
</tr>
<tr>
<td>Dolichol 19</td>
<td>0.10a</td>
<td>0.05a</td>
<td>0.10b</td>
<td>—</td>
</tr>
<tr>
<td>Dolichol 24</td>
<td>0.10a</td>
<td>0.05a</td>
<td>0.10b</td>
<td>—</td>
</tr>
<tr>
<td>Polypropenol 11</td>
<td>0.15a</td>
<td>0.10a</td>
<td>1.00c</td>
<td>10.00a</td>
</tr>
<tr>
<td>Polypropenol 12</td>
<td>0.15a</td>
<td>0.10a</td>
<td>1.00c</td>
<td>10.00a</td>
</tr>
<tr>
<td>Polypropenol 13</td>
<td>0.15a</td>
<td>0.10a</td>
<td>1.00c</td>
<td>10.00a</td>
</tr>
</tbody>
</table>

a [M + Li]+ ion was detected.
b [M + Li – (C_{5}H_{8})_{3}] ion was detected.  
c [M + Li – H_{2}O] ion was detected.  
d [M + Li – H_{2}O – (C_{5}H_{8})_{3}] ion was detected.

The polyisoprenoid standards were injected separately into the column using the inject valve of the mass spectrometer, and a chromatogram peak of each polyisoprenoid was obtained. A calibration curve was generated using the area of each peak in the chromatograms corresponding to the different concentrations of each polyisoprenoid standard (analytical standards), and the linear regression of the calibration curve, as well as the signal/noise (S/N) ratio for each of them, was calculated. The detection limits (defined as the concentration of the sample with an S/N ratio >3) are shown in Table 2.

Results and discussion

ESI(Li+)-MS of polyisoprenoids

ESI–MS analysis of polyisoprenoid alcohols as Na+ adducts was described previously [26], showing that analysis of polyisoprenoid compounds without extensive derivatization is possible. When we tried to apply ESI(Na+)–MS to these compounds in Leishmania and Plasmodium samples using sodium acetate, however, no [M + Na]+ adducts were observed. Therefore, we investigated the use of other salts to promote efficient ESI of the polyisoprenoid compounds (lithium acetate, sodium iodide, sodium iodate, and...
lithium iodide) to try to achieve the best S/N ratio. Lithium iodide gave the best results.

Different solvents for diluting samples were also tested, and chloroform:methanol (1:1, v/v) provided the best results. The solvent systems most often employed for chromatography of polyisoprenoids, however, provide inferior ESI(Li⁺)–MS sensitivity compared with chloroform:methanol (1:1, v/v).

Fig. 1 shows ESI(Li⁺)–MS (in the m/z region near that of the expected Li⁺ adduct) for 1-μM standard solutions of dolichol 11 (Fig. 1A) and polyrenol 11 (Fig. 1B) spiked with 5 mM of lithium iodide. In these spectra, the singly charged lithium adducts [M + Li⁺] of m/z 776 and m/z 774 are clearly observed for dolichol and polyrenol of 11 isoprene units, respectively. These spectra indicate, therefore, that ESI(Li⁺)–MS of polyisoprenoids is indeed feasible and efficient and that this “single ion” detection improves sensitivity and facilitates mixture analysis. It should also be noted that the protonated molecules [M + H⁺] in both ESI(Li⁺)–MS are absent.

ESI(Li⁺)–MS efficiency is dependent on the lithium iodide concentration because high concentrations promote ion suppression. Therefore, to achieve the highest ESI(Li⁺)–MS efficiency for polyisoprenoids, variable lithium iodide concentrations (1–20 mM) were tested for the standards of dolichol and polyrenol of 11 isoprene units. Solutions were analyzed separately and injected continuously into the ESI source at a flow rate of 10 μl/min.

ESI(Li⁺)–MS spectra were acquired along the m/z range shown in Table 1, and ESI(Li⁺)–MS spectra over a more restricted range (m/z 800 ± 5) were also acquired to measure background noise. Highest sensitivity was obtained with lithium iodide at a concentration of 2–5 mM (data not shown); hence, all further ESI(Li⁺)–MS spectra were acquired using lithium iodide at a concentration of 2 mM.

To evaluate the efficiency of ESI(Li⁺)–MS in analyzing polyisoprenoids with different isoprene chain sizes, polyisoprenoid standards (geranylgeraniol, dolichol 24, and a mixture of polyrenols of 10, 11, and 12 isoprene units) at 1-μM concentrations were tested (Figs. 1C, D, and E, respectively). ESI(Li⁺)–MS was found to be efficient regardless of the chain size. As Fig. 1E shows, the three isoprenoids in a mixture (polyrenols of 10, 11, and 12 isoprene unit standards) were ionized with an efficiency similar to that of their [M + Li⁺] adducts.

To calculate the ESI(Li⁺)–MS and ESI(Li⁺)–MS/MS LOD (Table 2), standards of geraniol, geranylgeraniol, dolichols of 11, 19, and 24 isoprene units, and polyrenols of 8, 9, 10, 11, and 12 isoprene units were analyzed both in “full ion” mode and via SIM. Table 2 shows that concentrations as low as 100 nM can be detected in the full ion mode for dolichols 19 and 24, and as expected, even better results are obtained using SIM (50 nM for dolichols 19 and 24). Therefore, ESI(Li⁺)–MS is a sensitive method for polyisoprenoid detection. Because the measurements were performed in an ion trap instrument, we expect that even better LODs could be achieved using SIM in quadrupole mass spectrometers.

EI–MS of polyisoprenoids [17] produces protonated molecules that, when dissociated, produce characteristic fragments that allow structural investigation. However, the dissociation of Li⁺ adducts of polyisoprenoids has not yet been studied.
Fig. 2 shows ESI(Li⁺)-MS/MS of [M + Li⁺]⁺ ions of m/z 776 from dolichol 11 (Fig. 2A) and m/z 1661 from dolichol 24 (Fig. 2B). A series of very structurally diagnostic fragment ions corresponding to sequential loss of 68-Da isoprene units (C₅H₈) are evident ([M + Li - (C₅H₈)]⁺) (Scheme 3A). A series of [M + Li - (C₅H₈)ₙ - H₂O]⁺ fragments initiated by water loss (the [M + Li - H₂O]⁺ of m/z 758 and m/z 1643) are also detected but in lower abundances.

For polyprenols, unlike dolichols, ESI(Li⁺)-MS/MS of [M + Li⁺]⁺ detects mainly a single-fragment ion as a result of water loss (Fig. 2C). As Fig. 2C shows for the polyprenol of 11 isoprene units, although water loss from the [M + Li⁺]⁺ adduct of m/z 774 forming [M + Li - H₂O]⁺ of m/z 756 is the main process, a minor [M + Li - CH₂O]⁺ of m/z 744 is also detected. The same dissociation pattern was observed for all other polyprenols tested. Therefore, this contrasting...
dissociation behavior differentiates between dolichols and polyisoprenoids.

Although ESI(Li+)–MS/MS of the [M + Li]+ adducts of polyisoprenoids shows water loss as the major process and thus provides limited structural information, ESI(Li+)–MS3 of the [M + Li – H2O]+ fragment retrieves the missing structural information. This is because ESI(Li+)–MS3 of polyisoprenoids displays the same series of structurally diagnostic fragment ions as a result of sequential loss of 68-Da isoprene units (Fig. 2D) (i.e., [M + Li – (C₅H₈)n – H₂O]+) as does ESI(Li+)–MS/MS of dolichols (i.e., [M + Li – (C₅H₈)n]+).

Dissociation pathways

The structural difference between dolichols and polyisoprenoids is the additional double bond in the last isoprene unit in polyisoprenoids (Scheme 1). The much more pronounced proclivity of Li+ adducts of polyisoprenoids to lose water under collision-induced dissociation (CID), therefore, is likely to be driven by this double bond. Scheme 3B presents a possible dissociation mechanism for water loss from polyisoprenols. A hydrogen atom is transferred to the hydroxyl group via a favored six-member ring in a process similar to the McLafferty rearrangement for radical cations [34]. Thus, the water loss product formed bears conjugated double bonds that should enhance stability. Similar water loss is not possible for dolichols; hence, they dissociate preferentially by the consecutive loss of 68-Da isoprene units (C₅H₈) (Scheme 3A). For some small polyisoprenoids, OH loss was also observed, and although the loss of this radical is against the even-electron rule [35], it forms a stable allylic radical. Again, the presence of the double bond in the β-position permits allylic radicals to be formed on OH loss, making this fragmentation exclusive to polyisoprenols (Scheme 3B).

Characterization of dolichols of 11 and 12 isoprene units from L. amazonensis

After establishing the best conditions for ESI(Li+)–MS of polyisoprenoids, we used this method to characterize dolichols in L. amazonensis. Hexane extracts of Leishmania promastigotes were purified by HPLC (described in Materials and methods), and the fractions with the same retention times as standards of dolichol with 11 isoprene units and polyisoprenol of 12 isoprene units were analyzed by ESI(Li+)–MS and ESI(Li+)–MS/MS.

A dolichol of 11 isoprene units was indeed detected in the sample purified from L. amazonensis promastigotes as a major [M + Li]+ ion of m/z 775.7 (spectrum not shown). The same [M + Li]+ adduct was also detected when the authentic dolichol standard of 11 isoprene units was subjected to ESI(Li+)–MS. The molecular identity was confirmed by comparing the ESI(Li+)–MS/MS of the ion of m/z 775.7 from L. amazonensis promastigotes (Fig. 3B) with that of the standard (Fig. 3A). In both spectra, the same structurally diagnostic dissociation profile with successive loss of isoprene units (68 Da) is evident.

In addition, a dolichol composed of 12 isoprene units was detected in the sample purified from L. amazonensis promastigotes as [M + Li]+ of m/z 844.6 (spectrum not shown).
shown). Due to the lack of a dolichol standard of 12 isoprene units (which is commercially not available), structural characterization via ESI(Li+)–MS/MS comparison was not possible. Therefore, this characterization was based solely on the interpretation of the dolichol’s ESI(Li+)–MS/MS dissociation chemistry. As shown in Fig. 3C, this [M + Li]+ adduct displays the same dissociation pattern as dolichol 11, namely sequential losses of C5H8, forming mainly the fragment ions of m/z 501.3 [M + Li − 274 Da]+, 433.1 [M + Li − 342 Da]+, 365.5 [M + Li − 410 Da]+, 298.5 [M + Li − 478 Da]+, and 230.2 [M + Li − 546 Da]+, demonstrating for the first time the presence of dolichol molecules in *L. amazonensis* [36].

**Characterization of dolichols of 11 and 12 isoprene units from *P. falciparum***

In 1999, our group described the presence of dolichols of 11 and 12 isoprene units in *P. falciparum* [37] using metabolic labeling with radioactive isotopes and high-performance thin-layer chromatography (HPTLC) techniques. Once the ESI(Li+)–MS technique for polyisoprenoids had

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**Fig. 3.** ESI(Li+)–MS/MS of [M + Li]+ ion of m/z 775.8 of dolichol standard of 11 isoprene units (A), [M + Li]+ of m/z 775.7 from *L. amazonensis* (B), and [M + Li]+ of m/z 844.3 from *L. amazonensis* (C).

**Fig. 4.** (A1,B1) ESI(Li+)–MS of the dolichol of 11 isoprene units (m/z 775.9) and 12 isoprene units (m/z 844.2), respectively, from *P. falciparum* schizonts. (A2,B2) ESI(Li+)–MS/MS of [M + Li]+ ion of m/z 775.9 of dolichol of 11 isoprene units and [M + Li]+ of m/z 844.2 of dolichol of 12 isoprene units, respectively.
been established, we tried to confirm the identity of the dolichols in *P. falciparum* using this new ESI–MS technique.

Hexane extracts of *Plasmodium* schizonts were purified by HPLC, and the fractions with the same retention time as standards of dolichol of 11 isoprene units and polyprenol of 12 isoprene units were analyzed by ESI(Li⁺)–MS and ESI(Li⁺)–MS/MS.

A dolichol of 11 isoprene units was indeed detected as a major [M + Li]⁺ ion of *m/z* 775.9 in the sample purified from schizonts (Fig. 4A1). The same [M + Li]⁺ adduct was also detected when the authentic dolichol standard of 11 isoprene units was subjected to ESI(Li⁺)–MS (Fig. 1A) and its molecular structure was confirmed by comparing the ESI(Li⁺)–MS/MS of the ion of *m/z* 775.9 from *P. falciparum* (Fig. 4A2) with that of the standard (Fig. 2A). The same structurally diagnostic dissociation profile with successive loss of isoprene units (68 Da) is evident.

A dolichol of 12 isoprene units was also detected as [M + Li]⁺ of *m/z* 844.2 (Fig. 4B1). As in the *Leishmania* analysis, structural characterization via ESI(Li⁺)–MS/MS by comparison with a dolichol standard was not possible due to the lack of a dolichol standard of 12 isoprene units (which is commercially not available) and, therefore, was based solely on the interpretation of the dolichol’s ESI(Li⁺)–MS/MS dissociation chemistry. As shown in Fig. 4B2, this [M + Li]⁺ ion displays the same dissociation pattern as dolichol 11, namely sequential losses of C₅H₈ (68 Da), showing that this molecule is really a dolichol of 12 isoprene units.

**Characterization of octaprenyl pyrophosphate synthase products in *P. falciparum***

Octaprenyl pyrophosphate synthase (OPPs) belongs to a prenyltransferase family that catalyzes the condensation
reaction of farnesyl pyrophosphate with five molecules of isopentenyl pyrophosphates to produce a 40-carbon octaprenyl pyrophosphate product [38].

Our group cloned the gene encoding a putative TPIOPPs characterized its gene product and obtained a recombinant version of this protein [32]. In parallel, a purified native version of this enzyme (PrOPPs) from schizont forms was obtained, and its functional and kinetic properties were compared with those of the recombinant version [32].

The polypropenyl products catalyzed by PrOPPs were separated by HPLC into their dephosphorylated forms, and the fractions corresponding to peaks with retention times of 25, 26, and 30 min coincident with the authentic polypropenyl standards of 8, 9, and 11 isoprene units were analyzed by ESI(Li⁺)–MS in full ion and SIM modes as well as by ESI(Li⁺)–MS/MS.

The presence of a polypropenol of 8 isoprene units represented by the [M + Li]⁺ ion of m/z 569.4, a polypropenol of 9 isoprene units represented by the [M + Li]⁺ ion of m/z 637.6, and a polypropenol of 11 isoprene units represented by the [M + Li]⁺ ion of m/z 773.9 (Figs. 5A1, B1, and C1, respectively) was observed in the reaction with TPIOPPs.

These same ions were also detected when the authentic polypropenol standards of 8, 9, and 11 isoprene units were analyzed (spectrum not shown). Structural characterization was confirmed by the great similarity between the ESI(Li⁺)–MS/MS of the [M + Li]⁺ adduct (Figs. 5A2, B2, and C2) and that of the standard (spectrum not shown), showing a minor [M + Li – CH₂O]⁺ fragment ion and a major [M + Li – H₂O]⁺ fragment ion of m/z 551.2, m/z 619.4, and m/z 755.6 for polypropenols of 8, 9, and 11 isoprene units, respectively, characteristic of polypropenol molecules.

In the reactions with PrOPPs, only polypropenols of 8 isoprene units ([M + Li]⁺ adduct of m/z 569.3) and 11 isoprene units (represented by the [M + Li]⁺ ion of m/z 774.0) were detected (Figs. 5D1 and E1). Their structural characterization was confirmed by the great similarity between the ESI(Li⁺)–MS/MS of the [M + Li]⁺ adduct (Figs. 5D2 and E2) and that of the standard. The polypropenol of 9 isoprene units was not detected, indicating that its concentration was below the LOD for ESI(Li⁺)–MS (100 nM).

In both experiments with TPIOPPs and PrOPPs, ESI(Li⁺)–MS³ did not detect the [M + Li – (C₅H₈)n – H₂O]⁺ ions, indicating that the concentration of the polypropenols was below the detection limit for these experiments (10 nM).

Conclusions

In this study, we have described an effective new approach based on ESI(Li⁺)–MS and ESI(Li⁺)–MS/MS for the detection and structural investigation of polypropenoids. Both dolichols and polypropenols are ionized efficiently, and the [M + Li]⁺ adducts of dolichols dissociate extensively and preferentially by the sequential loss of 68-Da isoprene units, whereas polypropenols dissociate nearly exclusively by water loss. These characteristic and diverse dissociation patterns allow polypropenoid structures to be clearly identified and distinguished. ESI(Li⁺)–MS³ of the water loss fragment [M + Li – H₂O]⁺ from polypropenols also shows the characteristic series of isoprene (68 Da) loss fragments, thereby recovering this structural information missing from the ESI(Li⁺)–MS/MS data. Using this method, polypropenoids of different chain sizes can be detected with high sensitivity, that is, an LOD of approximately 50 nM. The experiments with Leishmania and Plasmodium samples also demonstrated that ESI(Li⁺)–MS and ESI(Li⁺)–MS/MS can be applied to detect and characterize polypropenoids at trace levels in complex mixtures, thereby greatly facilitating the screening of such compounds in various matrices.

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