

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 $[\text{M} + \text{Li}]^+$ adducts. Thus, detection of polyisoprenoids with mass determination can be performed with high sensitivity (limit of detection [LOD] $\sim 100 \mu\text{M}$), 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*.

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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 α -saturated isoprenoid alcohols found in all animal cells and some bacteria, parasites, fungi, and plants, and (ii) polyprenols (Scheme 1B), which are α -unsaturated isoprenoid alcohols found in the green tissues of many plants, bacteria, yeast, and parasites.

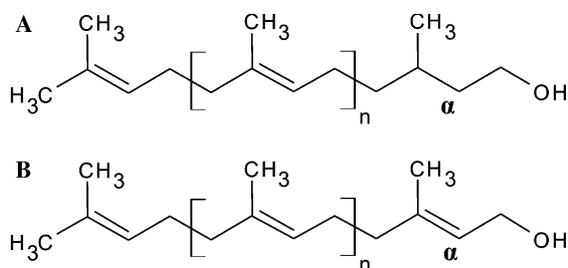
Animals, bacteria, and some parasites such as *Leishmania amazonensis* are known to biosynthesize isoprenoids via the mevalonic acid pathway [3,4], whereas plants

and some bacteria and parasites such as *Plasmodium falciparum* use the 2-C-methyl-D-erythritol 4-phosphate (MEP)¹ pathway [5,6]. These two pathways have been found to occur concurrently in organisms such as bacteria and fungi [7].

¹ Abbreviations used: MEP, 2-C-methyl-D-erythritol 4-phosphate; GPI, glycosylphosphatidyl inositol; EI–MS, electron ionization mass spectrometry; TBDMS, *tert*-butyl-dimethylsilyl; FAB–MS, fast atom bombardment mass spectrometry; FD–MS, field desorption mass spectrometry; ESI–MS, electrospray ionization mass spectrometry; ESI(Li^+)–MS/MS, ESI(Li^+) tandem mass spectrometry; PBS, phosphate-buffered saline; PfOPPs, *P. falciparum* octaprenyl pyrophosphate synthase; TPfOPPs, truncated recombinant *P. falciparum* octaprenyl pyrophosphate synthase; FPP, farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate; SIM, selective ion monitoring; LOD, limit of detection; LC–MS, liquid chromatography mass spectrometry; S/N, signal/noise; CID, collision-induced dissociation; HPTLC, high-performance thin-layer chromatography; OPs, octaprenyl pyrophosphate synthase.

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Scheme 1. Structure of dolichols (A) and polyprenols (B). α -Terminal isoprene unit.

Polyprenols and dolichols are found in cells as free alcohols and esters [8]. Phosphorylated polyisoprenoids 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 groups for protein prenylation [11].

The main biological role of free polyisoprenoid 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 polyisoprenoids in biological samples [16].

In electron ionization mass spectrometry (EI-MS) analysis [17,18], underivatized polyisoprenoids 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 polyisoprenoids with EI-MS, off-line derivatization to *tert*-butyl-dimethylsilyl (TBDMS) ethers [17] normally is used. The hydroxyl groups of polyisoprenoids 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 polyisoprenoid 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 anal-

ysis to increase polarity and facilitate protonation or deprotonation of the molecules.

Due to the high hydrophobicity of the polyisoprenoid 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 polyisoprenoids 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 polyisoprenoid alcohols occurs efficiently via Li^+ cationization, that is, via their $[\text{M} + \text{Li}]^+$ 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 polyisoprenoid 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%), geranylgeraniol ($\geq 85\%$), 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 ($\geq 98.0\%$) 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% CO_2 , 4.93% O_2 , and 90.20% N_2 . Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears.

Cultures ($\sim 20\%$ 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 2000g, 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 Na_2HPO_4 , 0.01 M NaH_2PO_4 , pH 7.4, 0.15 M NaCl) at 10,000g 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 $\mu\text{g}/\text{ml}$ of heme, 40 mM Hepes (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, pH 7.4), 50 units/ml of penicillin, and 50 $\mu\text{g}/\text{ml}$ 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 LCD-Duo ion 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- $\mu\text{l}/\text{min}$ flow rate (Scheme 2C) or through an inject valve with a 10- μl loop using an OmniFit N_2 pressure system (OmniFit, Cambridge, UK) as a solvent pump set at 10 psi with a flow rate of 10 $\mu\text{l}/\text{min}$ (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.

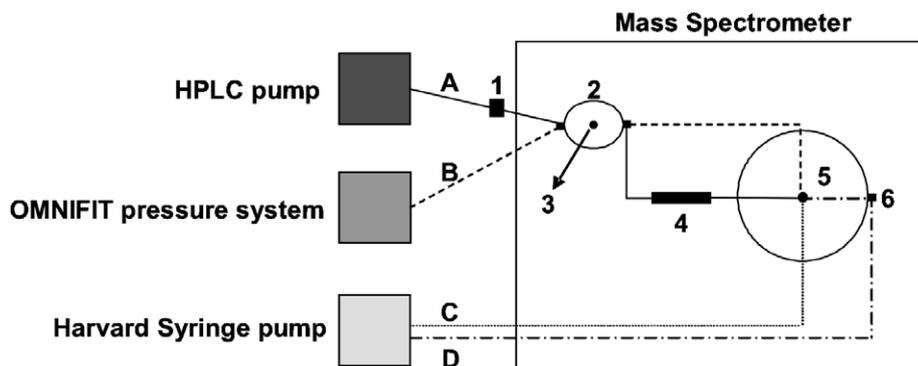
Extraction and preparation of samples

Leishmania amazonensis samples

To analyze dolichol from *L. amazonensis*, freeze-dried pellets of promastigote parasites ($\sim 1 \times 10^{11}$) 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 Ultrasphere ODS C18 Beckman column (250 mm \times 4.6 mm \times 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 polyprenol standards of 10, 11, and 12 isoprene units and



Scheme 2. Different methods for sample injection in the mass spectrometer. (A) The HPLC column (4) was placed between the inject valve (2) and ESI source (5) of the mass spectrometer. The samples were injected into the column using the inject valve of the mass spectrometer with a 10- μl loop (3), and the solvent was pumped using the HPLC pump. A splitter (1) was placed after the inject valve to reduce the flow rate to 50 $\mu\text{l}/\text{min}$. (B) Samples were injected directly into the ESI source (5) using the inject valve (2) with a 10- μl loop (3), and the solvent was pumped using the OmniFit pressure system. (C) Samples were injected directly into the ESI source (5) using a Harvard syringe pump apparatus. (D) The auxiliary solvent was injected directly into the ESI source across the auxiliary solvent entry (6) using a Harvard syringe pump apparatus.

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 \times 4.6 mm \times 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. [26]). 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 ($\sim 2 \times 10^{12}$) 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 polyisoprenoid products analyzed in this study were obtained from in vitro enzymatic reaction of a purified version of a native *P. falciparum* octaprenyl pyrophosphate synthase (PfOPPs) and a truncated recombinant form of this enzyme (TPfOPPs) with the substrates [32].

After enzymatic reaction in 0.3 ml of HKMT buffer (100 mM Hepes, pH 7.5, 50 mM KCl, 0.5 mM MgCl_2 , 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 PfOPPs) 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 \times 4.6 mm \times 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 (TPfOPPs and PfOPPs), samples

with the same retention times as the polyisoprenoid 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- $\mu\text{l}/\text{min}$ flow rate (Scheme 2C). 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 inject valve 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 Omni-Fit 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 (N_2) 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 polyisoprenoids 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

Table 1

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

Standard	Molecular formula	Formula weight	Singly charged lithium adduct ion mass	m/z range (full ion mode)	m/z range (selective ion monitoring)
Geranylgeraniol	$\text{C}_{20}\text{H}_{34}\text{O}$	290.48	297.38	50–500	297 ± 3
Polyprenol 8	$\text{C}_{40}\text{H}_{66}\text{O}$	562.95	569.85	400–600	570 ± 3
Polyprenol 9	$\text{C}_{45}\text{H}_{74}\text{O}$	631.06	637.96	550–700	637 ± 3
Polyprenol 10	$\text{C}_{50}\text{H}_{82}\text{O}$	699.18	706.08	650–900	706 ± 3
Polyprenol 11	$\text{C}_{55}\text{H}_{90}\text{O}$	767.30	774.20	700–850	774 ± 3
Dolichol 11	$\text{C}_{55}\text{H}_{92}\text{O}$	769.31	776.21	700–850	776 ± 3
Polyprenol 12	$\text{C}_{60}\text{H}_{98}\text{O}$	835.42	842.32	650–900	842 ± 3
Dolichol 12	$\text{C}_{60}\text{H}_{100}\text{O}$	837.43	844.33	750–900	844 ± 3
Dolichol 19	$\text{C}_{95}\text{H}_{156}\text{O}$	1314.24	1321.14	700–1400	1321 ± 3
Dolichol 24	$\text{C}_{120}\text{H}_{196}\text{O}$	1654.51	1659.71	1500–1800	1660 ± 3

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 ρM , 10 ρM , 100 ρM , 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 because the retention time for all polyisoprenoids is the same (10 min) but provides a chromatography peak for each polyisoprenoid and allows the LOD to be calculated. A Phenomenex Luna C18 column (250 mm \times 1.0 mm \times 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 $\mu\text{l}/\text{min}$ (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 $\mu\text{l}/\text{min}$) as auxiliary solvent (Scheme 2D). The final concentration of lithium iodide in the sample was 2 mM.

Table 2

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

Standard	Full ion mode	Selective ion monitoring	MS/MS	MS ³
Geraniol	0.25 ^a	0.10 ^a	1.00 ^b	—
Geranylgeraniol	0.20 ^a	0.10 ^a	1.00 ^b	—
Dolichol 11	0.15 ^a	0.10 ^a	1.00 ^b	—
Dolichol 19	0.10 ^a	0.05 ^a	0.10 ^b	—
Dolichol 24	0.10 ^a	0.05 ^a	0.10 ^b	—
Polyprenol 11	0.15 ^a	0.10 ^a	1.00 ^c	10.00 ^d
Polyprenol 12	0.15 ^a	0.10 ^a	1.00 ^c	10.00 ^d
Polyprenol 13	0.15 ^a	0.10 ^a	1.00 ^c	10.00 ^d

^a $[\text{M} + \text{Li}]^+$ ion was detected.

^b $[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n]^+$ fragment ions were detected.

^c $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ ion was detected.

^d $[\text{M} + \text{Li} - \text{H}_2\text{O} - (\text{C}_5\text{H}_8)_n]^+$ fragment ions were 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 $[\text{M} + \text{Na}]^+$ 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 polyprenol 11 (Fig. 1B) spiked with 5 mM of lithium iodide. In these spectra, the singly charged lithium adducts $[\text{M} + \text{Li}]^+$ of m/z 776 and m/z 774 are clearly observed for dolichol and polyprenol 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 $[\text{M} + \text{H}]^+$ 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 polyprenol of 11 isoprene units. Solutions were analyzed separately and injected continuously into the ESI source at a flow rate of 10 $\mu\text{l}/\text{min}$.

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 \pm 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 polyprenols 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 (polyprenols of 10, 11, and 12 isoprene unit standards) were ionized with an efficiency similar to that of their $[\text{M} + \text{Li}]^+$ 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 polyprenols 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 μM can be detected in the full ion mode for dolichols 19 and 24, and as expected, even better results are obtained using SIM (50 μM 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.

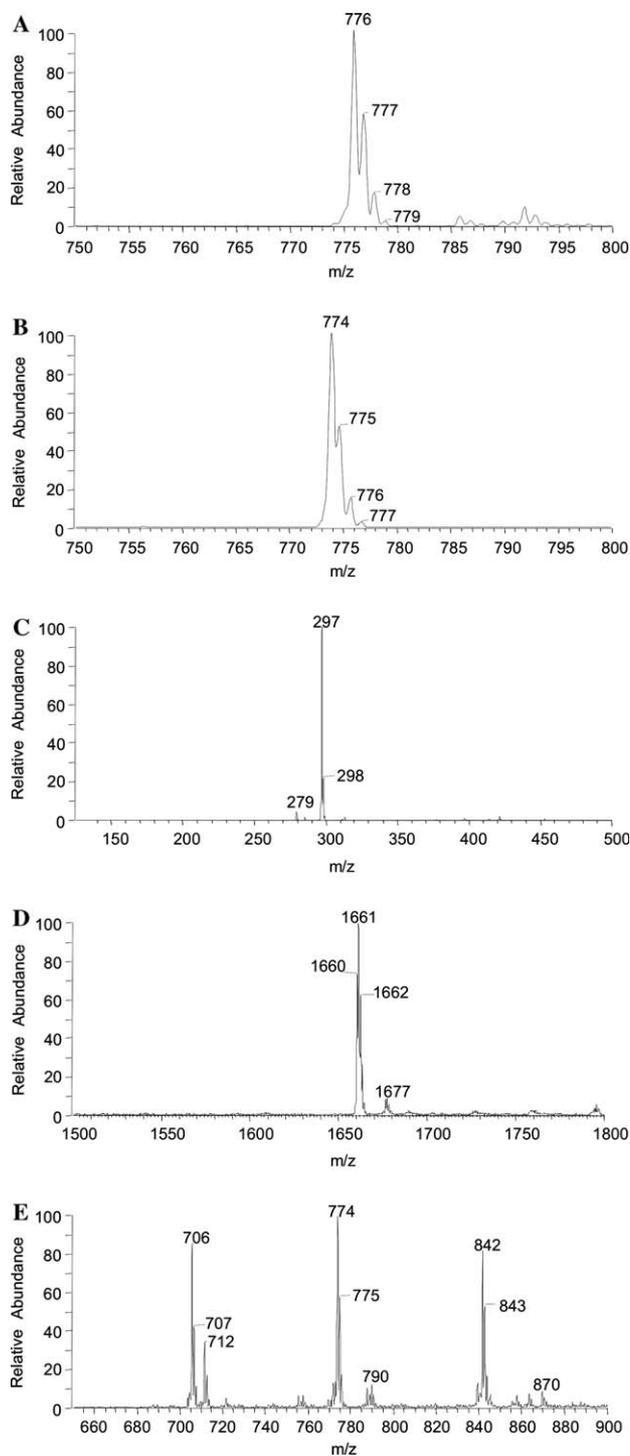


Fig. 1. ESI(Li^+)–MS of the solution of dolichol 11 (A), polyprenol 11 (B), geranylgeraniol (C), dolichol 24 (D), and a mixture of standards of polyprenols of 10, 11, and 12 isoprene unit standards (E).

ESI(Li^+)–MS/MS of dolichol and polyprenol standards

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 $[\text{M} + \text{Li}]^+$ 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 (C_5H_8) are evident ($[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n]^+$) (Scheme 3A). A series of $[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n - \text{H}_2\text{O}]^+$ fragments initiated by water loss (the $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ of m/z 758 and m/z 1643) are also detected but in lower abundances.

For polyprenols, unlike dolichols, ESI(Li^+)-MS/MS of $[\text{M} + \text{Li}]^+$ 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 $[\text{M} + \text{Li}]^+$ adduct of m/z 774 forming $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ of m/z 756 is the main process, a minor $[\text{M} + \text{Li} - \text{CH}_2\text{O}]^+$ of m/z 744 is also detected. The same dissociation pattern was observed for all other polyprenols tested. Therefore, this contrasting

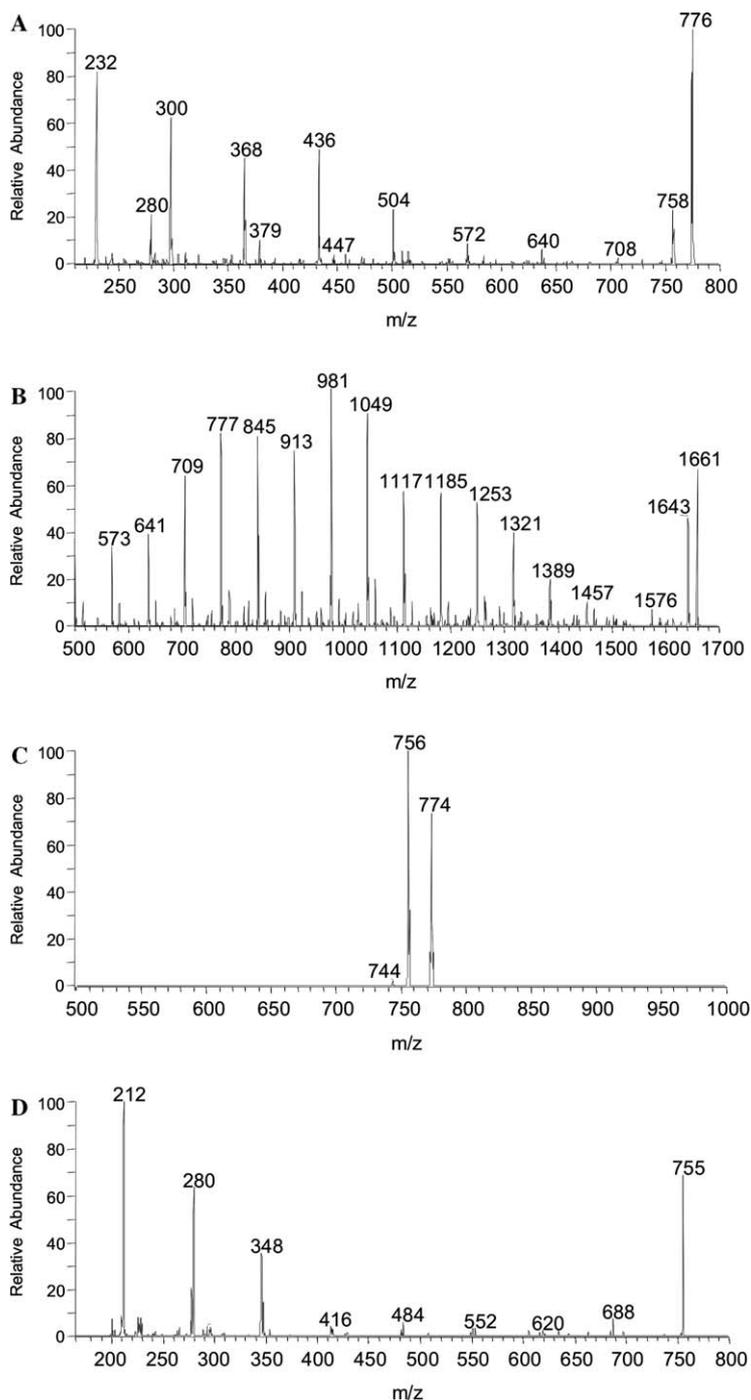


Fig. 2. (A,B) ESI(Li^+)-MS/MS of $[\text{M} + \text{Li}]^+$ of m/z 776 and m/z 1661 from 1- μM solution of dolichols of 11 and 24 isoprene units, respectively. (C,D) ESI(Li^+)-MS/MS and ESI(Li^+)-MS³, respectively, of $[\text{M} + \text{Li}]^+$ of m/z 774 and $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ of m/z 756 from 1- μM solution of polyprenol standard of 11 isoprene units.

dissociation behavior differentiates between dolichols and polyprenols.

Although $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ of the $[\text{M} + \text{Li}]^+$ adducts of polyprenols shows water loss as the major process and thus provides limited structural information, $\text{ESI}(\text{Li}^+)\text{-MS}^3$ of the $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ fragment retrieves the missing structural information. This is because $\text{ESI}(\text{Li}^+)\text{-MS}^3$ of polyprenols displays the same series of structurally diagnostic fragment ions as a result of sequential loss of 68-Da isoprene units (Fig. 2D) (i.e., $[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n - \text{H}_2\text{O}]^+$) as does $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ of dolichols (i.e., $[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n]^+$).

Dissociation pathways

The structural difference between dolichols and polyprenols is the additional double bond in the last isoprene unit in polyprenols (Scheme 1). The much more pronounced proclivity of Li^+ adducts of polyprenols 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 polyprenols. 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 (C_5H_8) (Scheme 3A). For some small polyprenols, OH loss was also observed, and although the loss of this radical is

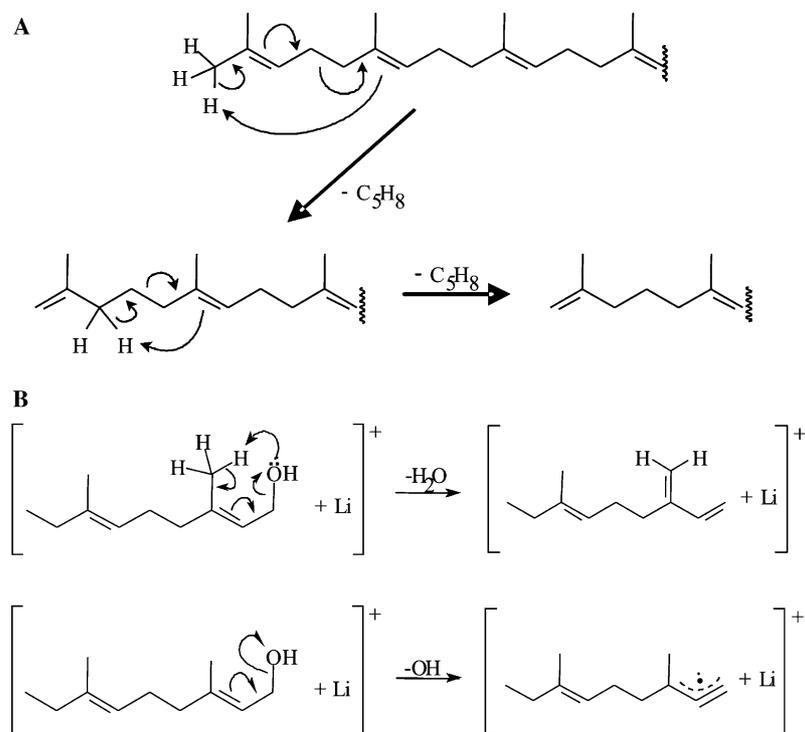
against the even-electron rule [35], it forms a stable allylic radical. Again, the presence of the double bond in the β -position permits allylic radicals to be formed on OH loss, making this fragmentation exclusive to polyprenols (Scheme 3B).

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

After establishing the best conditions for $\text{ESI}(\text{Li}^+)\text{-MS}$ 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 polyprenol of 12 isoprene units were analyzed by $\text{ESI}(\text{Li}^+)\text{-MS}$ and $\text{ESI}(\text{Li}^+)\text{-MS/MS}$.

A dolichol of 11 isoprene units was indeed detected in the sample purified from *L. amazonensis* promastigotes as a major $[\text{M} + \text{Li}]^+$ ion of m/z 775.7 (spectrum not shown). The same $[\text{M} + \text{Li}]^+$ adduct was also detected when the authentic dolichol standard of 11 isoprene units was subjected to $\text{ESI}(\text{Li}^+)\text{-MS}$. The molecular identity was confirmed by comparing the $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ 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 $[\text{M} + \text{Li}]^+$ of m/z 844.6 (spectrum not



Scheme 3. Rationalization of the dissociation pathways leading to consecutive losses of C_5H_8 (68 Da) from $[\text{M} + \text{Li}]^+$ adducts of both polyprenols and dolichols (A) and to water and OH loss from those of polyprenols (B).

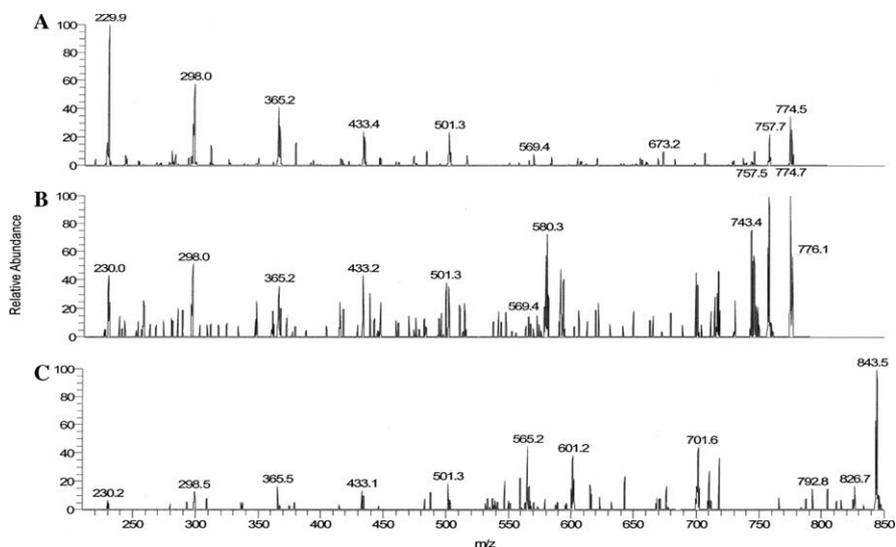


Fig. 3. ESI(Li^+)–MS/MS of $[\text{M} + \text{Li}]^+$ ion of m/z 775.8 of dolichol standard of 11 isoprene units (A), $[\text{M} + \text{Li}]^+$ of m/z 775.7 from *L. amazonensis* (B), and $[\text{M} + \text{Li}]^+$ of m/z 844.3 from *L. amazonensis* (C).

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 $[\text{M} + \text{Li}]^+$ adduct displays the same dissociation pattern as dolichol 11, namely sequential losses of C_5H_8 , forming mainly the fragment ions of m/z 501.3 $[\text{M} + \text{Li} - 274 \text{ Da}]^+$, 433.1 $[\text{M} + \text{Li} - 342 \text{ Da}]^+$, 365.5 $[\text{M} + \text{Li} - 410 \text{ Da}]^+$, 298.5 $[\text{M} + \text{Li} - 478 \text{ Da}]^+$, and 230.2 $[\text{M} + \text{Li} - 546 \text{ Da}]^+$, demon-

strating 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

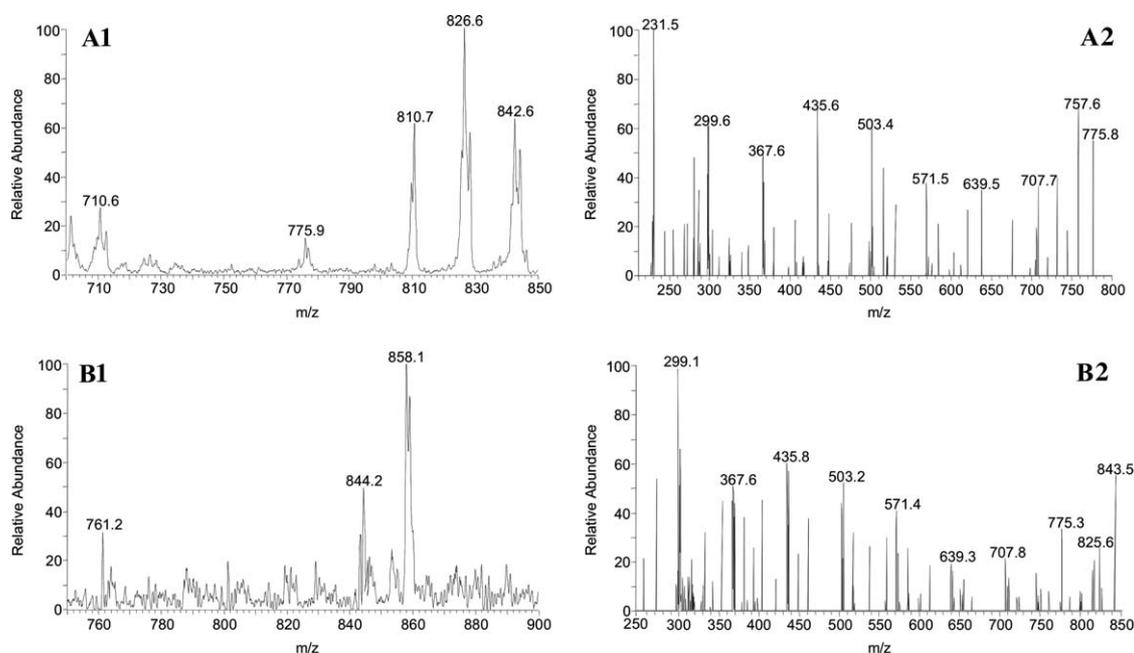


Fig. 4. (A1,B1) ESI(Li^+)–MS of the dolichol of 11 isoprene units (m/z 775.9) and 12 isoprene units (m/z 844.2), respectively, from *P. falciparum* schizonts. (A2,B2) ESI(Li^+)–MS/MS of $[\text{M} + \text{Li}]^+$ ion of m/z 775.9 of dolichol of 11 isoprene units and $[\text{M} + \text{Li}]^+$ of m/z 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 $[\text{M} + \text{Li}]^+$ ion of m/z 775.9 in the sample purified from schizonts (Fig. 4A1). The same $[\text{M} + \text{Li}]^+$ 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 $[\text{M} + \text{Li}]^+$ 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 $[\text{M} + \text{Li}]^+$ ion displays the same dissociation pattern as dolichol 11, namely sequential losses of C_5H_8 (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

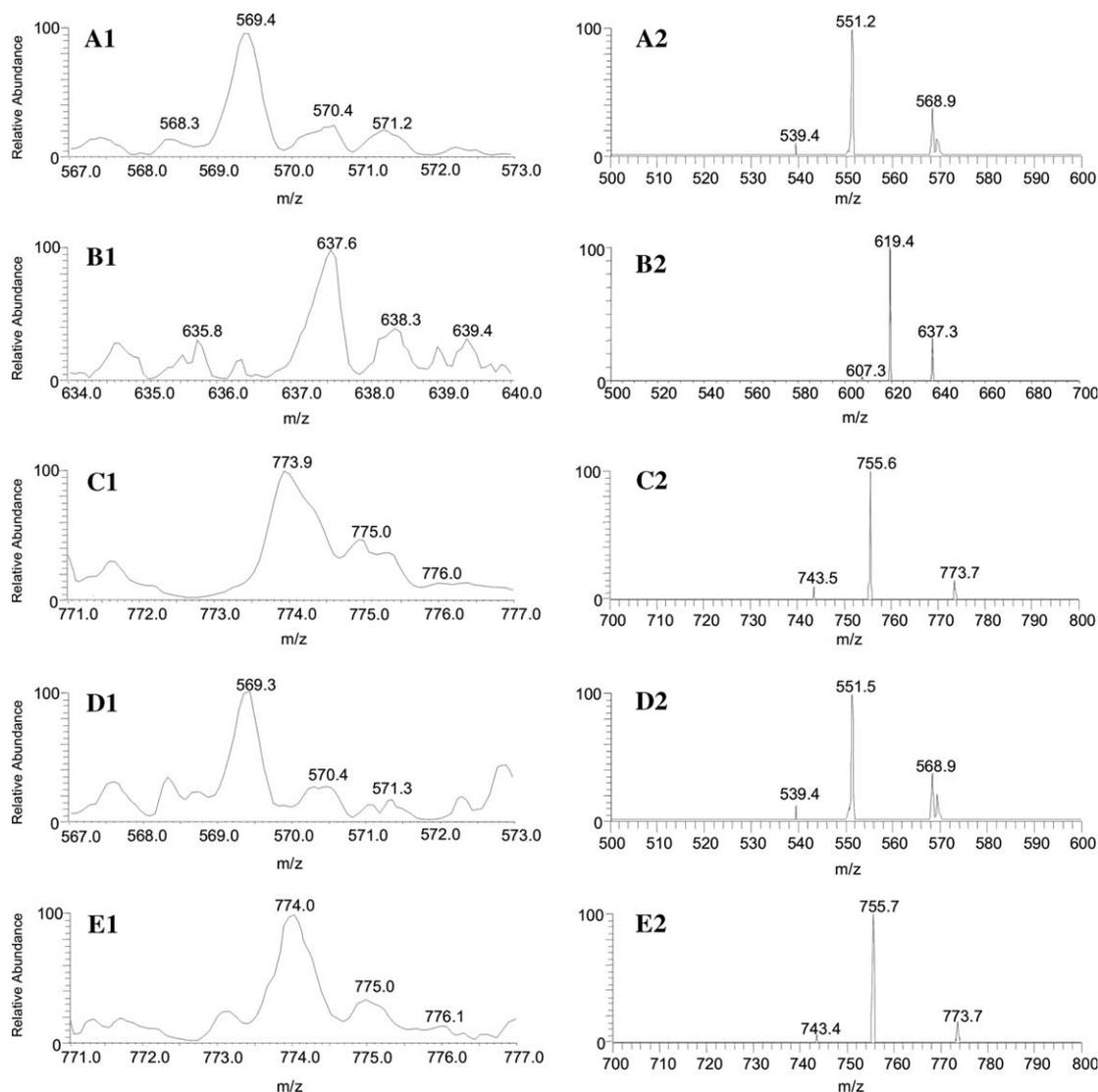


Fig. 5. (A1,B1,C1) ESI(Li^+)–MS of the polyprenols of 8 isoprene units (m/z 569.4), 9 isoprene units (m/z 637.6), and 11 isoprene units (m/z 773.9), respectively, from TPfOPPs reactions. (D1,E1) ESI(Li^+)–MS of the polyprenols of 8 isoprene units (m/z 569.3) and 11 isoprene units (m/z 774.0), respectively, from PfOPPs reactions. (A2,B2,C2) ESI(Li^+)–MS/MS of $[\text{M} + \text{Li}]^+$ of the polyprenols of 8, 9, and 11 isoprene units, respectively, from the TPfOPPs reactions. (D2,E2) ESI(Li^+)–MS/MS of $[\text{M} + \text{Li}]^+$ of the polyprenols of 8 and 11 isoprene units, respectively, from the PfOPPs reactions.

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

The polyprenol products catalyzed by PfOPPs 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 polyprenol standards of 8, 9, and 11 isoprene units were analyzed by $\text{ESI}(\text{Li}^+)\text{-MS}$ in full ion and SIM modes as well as by $\text{ESI}(\text{Li}^+)\text{-MS/MS}$.

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

These same ions were also detected when the authentic polyprenol standards of 8, 9, and 11 isoprene units were analyzed (spectrum not shown). Structural characterization was confirmed by the great similarity between the $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ of the $[\text{M} + \text{Li}]^+$ adduct (Figs. 5A2, B2, and C2) and that of the standard (spectrum not shown), showing a minor $[\text{M} + \text{Li} - \text{CH}_2\text{O}]^+$ fragment ion and a major $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ fragment ion of m/z 551.2, m/z 619.4, and m/z 755.6 for polyprenols of 8, 9, and 11 isoprene units, respectively, characteristic of polyprenol molecules.

In the reactions with PfOPPs, only polyprenols of 8 isoprene units ($[\text{M} + \text{Li}]^+$ adduct of m/z 569.3) and 11 isoprene units (represented by the $[\text{M} + \text{Li}]^+$ ion of m/z 774.0) were detected (Figs. 5D1 and E1). Their structural characterization was confirmed by the great similarity between the $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ of the $[\text{M} + \text{Li}]^+$ adduct (Figs. 5D2 and E2) and that of the standard. The polyprenol of 9 isoprene units was not detected, indicating that its concentration was below the LOD for $\text{ESI}(\text{Li}^+)\text{-MS}$ (100 ρM).

In both experiments with TPfOPPs and PfOPPs, $\text{ESI}(\text{Li}^+)\text{-MS}^3$ did not detect the $[\text{M} + \text{Li} - (\text{C}_5\text{H}_8)_n - \text{H}_2\text{O}]^+$ ions, indicating that the concentration of the polyprenols was below the detection limit for these experiments (10 nM).

Conclusions

In this study, we have described an effective new approach based on $\text{ESI}(\text{Li}^+)\text{-MS}$ and $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ for the detection and structural investigation of polyisoprenoids. Both dolichols and polyprenols are ionized efficiently, and the $[\text{M} + \text{Li}]^+$ adducts of dolichols dissociate extensively and preferentially by the sequential loss of 68-Da isoprene units, whereas polyprenols dissociate nearly

exclusively by water loss. These characteristic and diverse dissociation patterns allow polyisoprenoid structures to be clearly identified and distinguished. $\text{ESI}(\text{Li}^+)\text{-MS}^3$ of the water loss fragment $[\text{M} + \text{Li} - \text{H}_2\text{O}]^+$ from polyprenols also shows the characteristic series of isoprene (68 Da) loss fragments, thereby recovering this structural information missing from the $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ data. Using this method, polyisoprenoids of different chain sizes can be detected with high sensitivity, that is, an LOD of approximately 50 ρM . The experiments with *Leishmania* and *Plasmodium* samples also demonstrated that $\text{ESI}(\text{Li}^+)\text{-MS}$ and $\text{ESI}(\text{Li}^+)\text{-MS/MS}$ can be applied to detect and characterize polyisoprenoids at trace levels in complex mixtures, thereby greatly facilitating the screening of such compounds in various matrices.

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