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Analysis of endogenous ATP analogs and mevalonate pathway metabolites in cancer cell cultures using liquid chromatography–electrospray ionization mass spectrometry

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ABSTRACT

Nitrogen-containing bisphosphonates (N-BPs) are shown to inhibit a key enzyme of intracellular mevalonate pathway, FPP synthase, leading to intracellular accumulation of pathway metabolites isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In our previous studies we have shown that a new type of ATP analog, ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester), is also formed in addition to IPP and DMAPP accumulation. ApppI has cytotoxic effects leading to direct apoptosis of various cancer cells. In this study we present a validated method based on ion-pair LC–MS^2 for the analysis of isomeric mevalonate pathway metabolites and ATP analogs in cell culture samples. Limit of quantitation for IPP and DMAPP was 0.030 μM (1.35 fmol on-column) and for ApppI and ApppD 0.020 μM (0.9 fmol on-column). Acceptable accuracies and precision were also obtained for quality control samples in low and high concentrations of the calibration curve. In addition, we present a new method for quantitation of each coeluting isomer utilizing the peak intensity ratios of two characteristic fragment ions of each compound. For IPP and DMAPP, fragment ions *m*/*z* 177 and *m*/*z* 159 in the MS^2 were monitored, whereas for ATP analogs, ApppI and ApppD (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester), the same fragments in the MS^3 spectra were followed. IPP and DMAPP accumulation as well as ApppI and ApppD formation was demonstrated using MCF-7 breast cancer cells. Cells were treated with 25 μM zoledronic acid (an N-BP) for 24 h, conditions found to induce significant production of the metabolites. We found that the total amount of IPP and DMAPP was 2.4 nmol/mg of protein and amount of ApppI and ApppD was 1.1 nmol/mg protein. Relative portions of the isomers were approximately 1:4 IPP:DMAPP and 3:7 ApppI:ApppD. Untreated control samples did not contain IPP, DMAPP, ApppI or ApppD.

1. Introduction

The intracellular mevalonate pathway (MVP) produces several biomolecules essential for cell function, such as cholesterol, dolichol and respiratory quinines [1,2]. The metabolites of the pathway include isoprenoid pyrophosphates consisting of a five-carbon building block termed isoprene unit. The isoprenyl intermediates of the pathway are produced by sequential condensation reaction of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) yielding metabolites with carbon chain length of 10, 15 or 20 carbons, corresponding geranyl (GPP), farnesyl (FPP) and geranylgeranyl pyrophosphates (GGPP), respectively [3]. The chain elongation reactions are catalyzed by FPP and GGPP synthases [2]. FPP and GGPP are substrates for protein prenyltransferases catalyzing farnesylation and geranygeranylation reactions of small GTPases signaling proteins [4], enabling their trafficking to cell membranes [5].

MVP is a site of action for nitrogen-containing bisphosphonates (N-BPs), drugs used for treatment of various metabolic bone diseases (Fig. 1). These diseases express high bone resorption, medi-

Abbreviations: ANT, adenine nucleotide translocase; ApppD, triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester; ApppI, 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester; CID, collision induced dissociation; DMAPP, dimethylallyl pyrophosphate; DMHA, *N***N*-dimethylhexylamine; ESI, electrospray ionization; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; MVP, mevalonate pathway; N-BP, nitrogen-containing bisphosphonate; ZOL, zoledronic acid.

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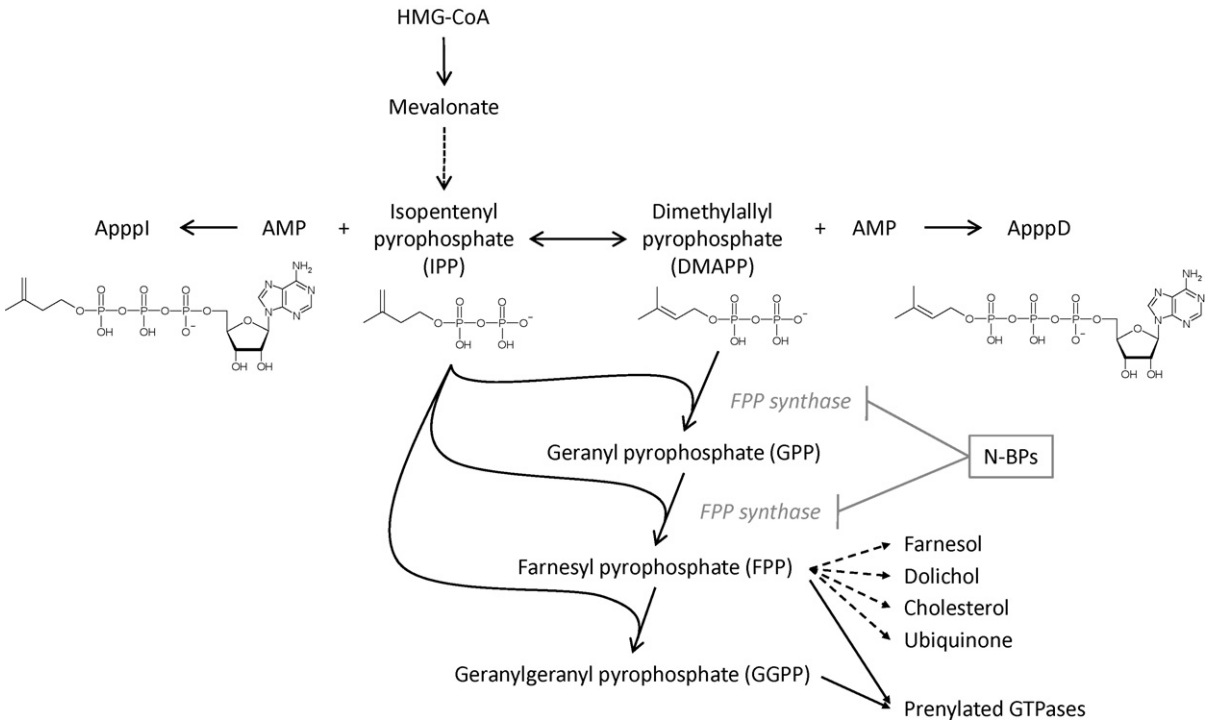


Fig. 1. Schematic presentation of the mevalonate pathway (MVP) and molecule structures of IPP, DMAPP, ApppI and AppppD.

ated by increase in either number or activity of osteoclasts [6–8]. The anti-resorptive activity of N-BPs is most likely due to their direct effect on mature osteoclasts [7]. Moreover, it is becoming evident that N-BPs also have an anti-tumorial effect *in vitro* and *in vivo* [9,10]. The molecular mechanism of action of the N-BPs (such as zoledronic acid, ZOL) is *via* inhibition of the FPP synthase, the key enzyme of the mevalonate pathway [11–13], in particular, due to consequent depletion of prenylated GTPases [14,15]. However, accumulation of the unprenylated GTPases and subsequent inappropriate activation of downstream signaling pathways may also account for the anti-resorptive effect of N-BPs rather than loss of the prenylated proteins [16–18]. Based on our recent findings we have proposed an additional mechanism of action for N-BPs. The inhibition of FPP synthase by N-BPs, in addition to depletion of the biosynthesis of FPP and GGPP, leads to accumulation of the early metabolites of mevalonate pathway, IPP and DMAPP [19–22]. The amount of these metabolites in excess of their physiological levels induces biosynthesis of a novel ATP analog ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester) as a conjugation reaction of IPP to 5′-adenosine monophosphate (AMP), catalyzed presumably by aminoacyl-tRNA-synthetases [19]. Our results show that ApppI causes apoptosis by blocking the mitochondrial adenine nucleotide translocase (ANT) and thus can evoke direct apoptosis. We have established the IPP accumulation and subsequent ApppI formation *in vitro* in J774 macrophage like cells after treatment with several N-BPs [19] as well as in different cancer cells after exposure to ZOL [20]. Also, similar effect of ZOL treatment was seen *in vivo* in mouse peritoneal macrophages [21].

Previously, the studies of bisphosphonate metabolism focused on analysis of an ATP analog metabolite of a non-N-BP clodronate, AppCCl$_2$p (adenosine 5′(β,γ-dichloromethylene)), in perchloric acid extracts of amoebae using anion-exchange fast protein liquid chromatography (FPLC) and UV detection [24,25]. Anion-exchange chromatography has also been a common method for analysis for other types of nucleotides and nucleotide derivatives [26–28], however, it is not a very suitable method when coupled with mass spectrometry. More sensitive and versatile methods have been developed for the analysis of AppCCl$_2$p utilizing ion-pair high-performance liquid chromatography (HPLC) and tandem mass spectrometry, which allows detection of several metabolites in a single chromatographic run [29,30]. Ion-pair chromatography is more favorable for mass spectrometric detection than anion-exchange since volatile reagents are used in the solvents. Several other HPLC methods have been described for the analysis of phosphorylated isoprenoids using ion-pair modifiers [31,32] or salt additives [33]. Also, ion-pair chromatography is commonly used for analysis of several types of nucleotides and derivatives [34–36]. Recently hydrophilic interaction liquid chromatography (HILIC) has often been applied in separation of highly polar compounds [37–39]. This method has an advantage of not including any salts or other additives, which might interfere with the electrospray ionization (ESI). Moreover, in HILIC analytes elute at high concentration of organic solvent, which favors the ESI process. However, among the methods presented in the literature, only few capable of resolving isoprenoid isomers are reported. Separation of IPP and DMAPP was achieved by analyzing the compounds as their corresponding monophosphates or alcohols, produced by hydrolysis using alkaline phosphatase or inorganic pyrophosphatase [40]. Another method utilized a cyclodextrin column and a long gradient system in the separation of the compounds [41].

In our previous studies, we have analyzed the total concentration of IPP and DMAPP as well as ApppI and AppppD (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester) mixtures in the cell culture samples [19–22]. Molecule structure of each compound is shown in Fig. 1. We refer here the mixture of the isomeric compounds as IPP/DMAPP and ApppI/AppppD. In this paper we describe an analysis method for the four metabolites observed in cultured breast cancer cells (MCF-7) after N-BP treatment. The method is capable of analyzing the concentrations of each of the compounds separately. This method is based on ion-pair liquid chromatographic separation following electrospray ionization tandem mass spectrometry (LC–MS^2 or LC–MS^3). Although the method

was not able to chromatographically separate IPP and DMAPP isomers, they were quantified by a novel method by utilizing their MS^2 spectra and different signal intensity ratio of two characteristic fragment ions. Similarly, amounts of ApppI and ApppD were determined based on signal intensity ratios in MS^3 spectra. It is important to separately analyze the isomers in order to monitor the reactions and enzyme activities of the mevalonate pathway. The method for analysis of total concentration of IPP/DMAPP as well as ApppI/ApppD mixtures in MCF-7 cell samples was also validated in this study.

2. Experimental

2.1. Chemicals

Water was purified in a MilliQ Gradient purification system (Millipore, Bedford, MA, USA) and HPLC-grade methanol (MeOH) was purchased from Labscan Ltd. (Dublin, Ireland). *N,N*-dimethylhexylamine (DMHA) was from Aldrich (Milwaukee, WI, USA), isopentenyl pyrophosphate (IPP), 3,3-dimethylallylpyrophosphate (DMAPP), methyleneadenosine 5′-triphosphate (AppCp), bovine serum albumin and sodium orthovanadate (Na_3VO_4) were from Sigma Chemical Co. (St. Louis, MO, USA) and sodium fluoride (NaF) from Riedel-de-Haën (Seelze, Germany). Zoledronic acid [2-(imidazol-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt, 4.75 hydrate] was kindly provided by Novartis Pharma AG (Basel, Switzerland). Stock solution of zoledronic acid was prepared in phosphate-buffered saline pH 7.4 (Gibco, Paisley, UK). Triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) was synthesized at the Department of Chemistry at the University of Kuopio [19].

2.2. Biological experiments

The experiments were performed using secondary cell line. The human estrogen-dependent breast cancer cell line, MCF-7 was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). Cells were cultured at 37 °C in a 5% CO_2 atmosphere in RPMI-1640 medium with L-glutamine (BioWhittaker, Cambrex Bioscience, Verviers, Belgium), supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen, Paisley, UK). Cells were harvested using 0.25% trypsin (Invitrogen). Plastics were supplied from Nunc (Thermo Fisher Nunc, Roskilde, Denmark).

Cells were seeded to a 6-well plate (1×10^6 cells/well) and allowed to adhere overnight. The following day the non-adherent cells were removed and the cells were treated with either 3 mL of fresh medium (control sample) or with 3 mL of fresh medium containing 25 μM ZOL for 24 h. After the treatment, medium was removed and the cells were washed with cold PBS.

To prepare the standard curve in untreated cell extracts, we also prepared samples similar to control sample to be used for this purpose.

2.3. Sample preparation

Sample preparation was adapted from previous method [42]. In short, to extract the analytes from the cell samples, ice-cold acetonitrile (300 μL) was pipeted to the cell culture plate with subsequent addition of ice-cold MilliQ-water (200 μL). The samples were collected by scraping the cells to the extraction solution, the plate was washed with 50 μL of water/acetonitrile (2:3, v/v) and the solutions were combined. Cell extracts were transferred to microcentrifuge tubes and suspended well by pipette mixing. Precipitated macromolecules were separated by centrifugation (13,000 × *g*, 3 min, 4 °C). The supernatant extract was transferred to a new tube and evaporated using vacuum centrifugation. Samples were either analyzed directly or stored at −20 °C until LC–MS analysis. The extraction procedure lasted in total 20 min and evaporation approximately 3 h. Prior to analysis, the samples were dissolved by vortex mixing in 150 μL of MilliQ-water containing 0.25 mM NaF and Na_3VO_4 phosphatase inhibitors and 1.0 μM AppCp as an internal standard, centrifuged (13,000 × *g*, 2 min) and the supernatant was transferred to LC analysis tubes.

The cell precipitates were digested with 1 mL of 1 M NaOH at 60 °C for 2 h with intermittent vortexing and analyzed for total protein content by a modified Bradford procedure (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard protein. Absorbance of protein samples were measured by Wallac Victor^2 Multilabel counter at 595 nm. The analyte concentrations are presented as nanomoles per milligram of protein (nmol/mg).

2.4. Liquid chromatography

The liquid chromatographic separation was carried out with a system comprised of an Agilent 1200 Series Binary Pump SL pump system and an Agilent 1200 Autosampler (Agilent Technologies, Inc., Santa Clara, CA, USA) or a Finnigan Surveyor MS pump and a Finnigan Surveyor autosampler (Thermo Electron Corporation, San Jose, CA, USA). The reversed phase column used was Phenomenex Gemini C18 (50 mm × 2.00 mm, 5 μm) with a similar guard column (4 mm × 2.00 mm) (Phenomenex, Torrance, CA, USA), column oven temperature was set at 20 °C and autosampler temperature at 4 °C. Injection volume was 45 μL and no-waste injection mode was used with the Finnigan LC system. Eluent flow rate was 200 μL/min and the eluents were 20 mM DMHA formate with pH adjusted to 6.8 with formic acid and 80% methanol containing 2 mM DMHA formate. The solvents were filtered using 0.45 μm filters before use and stored at 4 °C if needed. The LC gradient was from 0% to 80% of methanol in 3 min where it remained for 4 min. The gradient was then decreased from 80% to 0% of methanol in 1 min and the column was stabilized by 5 min flow of 20 mM DMHA.

2.5. Mass spectrometry

An Agilent 6410 Triple Quad LC/MS and a Finnigan LTQ linear ion trap mass spectrometers were used for detection of the analytes. Both instruments were equipped with an electrospray ionization source and operated on negative ion mode. Both instruments were also programmed with divert valve to direct the eluent flow to the mass spectrometer from 3 min to 10 min. Generally, the triple quadrupole instrument was used for analysis of total concentration of IPP/DMAPP and ApppI/ApppD, whereas the ion trap instrument was applied to the separate analysis of each isomer.

Flow injection analysis was used to optimize the parameters of the mass spectrometers. For the Agilent triple quadrupole instrument optimal source parameters were following: drying gas temperature 300 °C, gas flow 8 mL/min, nebulizer gas pressure 40 psi and capillary voltage −4500 V. Negative full scan mass spectra were recorded at the mass range of *m*/*z* 60–650 after optimization of source parameters. Highest parent ion abundance was found using fragmentor voltages of 120 V for IPP/DMAPP, and 140 V for ApppI/ApppD and internal standard (ISTD) AppCp. Product ion (MS^2) spectra were generated using collision induced dissociation (CID) in the collision cell with nitrogen gas. Most intense product ion signal was achieved by applying offset voltages of 15 eV, 27 eV and 30 eV for IPP/DMAPP, ApppI/ApppD and AppCp, respectively. Following transitions were selected for multiple reaction monitoring (MRM): *m*/*z* 245 → 79 for IPP/DMAPP, *m*/*z* 574 → 227 for ApppI/ApppD and *m*/*z* 504 → 157 for AppCp. Data acquisition was performed using Agilent MassHunter Workstation software.

The measurements with the LTQ ion trap were carried out using following parameters: the spray was stabilized with nitrogen sheath flow with value set to 30 (instrument units), spray needle voltage was −7000 V, the stainless steel capillary temperature 270 °C, capillary voltage −7 V and the tube lens offset −90 V. The spectra were recorded using 80 ms for collection of the ions in the trap. Collision energy was 30 V for both MS^2 and MS^3 and isolation width was 2.5 for IPP/DMAPP and 2.0 for ApppI/ApppD and AppCp. Selected MS^2 and MS^3 transitions were m/z 245 → 159 and m/z 245 → 177 for IPP and DMAPP, m/z 574 → 245 → 159 and m/z 574 → 245 → 177 for ApppI and ApppD, and m/z 504 → 406 for AppCp. Data acquisition was performed using Xcalibur 1.4 SR1 software and quantitation of the analytes using LCquan 2.0 quantification software.

2.6. Assay validation

2.6.1. Quantitation of the total amounts of IPP/DMAPP and ApppI/ApppD

The assay for the analysis of total concentration of IPP/DMAPP and ApppI/ApppD mixtures was validated using IPP and ApppI standards. The limit of quantitation (LOQ) was determined as the lowest concentration with the percent relative standard deviation (RSD %) value lower than 20% and signal-to-noise ratio greater than 10:1. Calibration and quality control (QC) samples were prepared fresh daily from stock solutions. The calibration area ranged from 0.030 μM to 15.0 μM for IPP and from 0.020 μM to 15.0 μM for ApppI. The calibration curve was generated by three replicate measurements and nine standard points. The peak area ratios of the MRM chromatograms of the analytes and internal standard were plotted against the concentration of the standards. A linear $1/x$ fit, where x is concentration, was employed. QC samples were prepared at low, middle and high concentration range of the standard curve, e.g. at 0.045 μM, 0.30 μM, 0.60 μM and 12.0 μM for IPP and at 0.030 μM, 0.20 μM, 0.60 μM and 12.0 μM for ApppI.

Accuracy was reported as the percentage of the expected concentration and precision as a percent relative standard deviation (RSD %). Intra-day accuracy and precision were evaluated by six (n = 6) replicate measurements of the QC samples on the same day. Inter-day accuracy and precision were obtained by analysing the QC samples in three or six replicate injections on three different days (n = 9–12) over period of one week.

Matrix effect was determined by spiking six (n = 6) untreated MCF-7 cell extracts with known amount (1.0 μM) of IPP, ApppI and AppCp and comparing the obtained peak areas to the ones of matrix free standard of same concentration. Spiked sample also contained the phosphatase inhibitors (0.25 mM NaF and Na_3VO_4).

The recovery and precision of the extraction method were determined by analyzing the total content of IPP/DMAPP and ApppI/ApppD in six (n = 6) MCF-7 cell samples, treated with 25 μM ZOL for 24 h. Extraction recovery was obtained by repeating the extraction procedure twice for the cell precipitates.

2.6.2. Quantitation of the isomers

IPP and DMAPP in the ZOL treated cell samples were quantified using a calibration curve generated by preparing calibration standards containing variable percentages (0–100%) of IPP and DMAPP. The MRM chromatogram peak area ratio of m/z 245 → 177 and m/z 245 → 159 was plotted against the amount of IPP. The calibration samples were prepared in water and analyzed in three replicate instrumental measurements. QC samples for evaluation of accuracy and precision were also prepared in water in three different compositions of IPP and DMAPP. The total concentration of IPP and DMAPP in the calibration and QC samples was 1.0 μM. Samples containing equal concentrations of IPP and DMAPP (0.25 μM, 1.0 μM and 5.0 μM) were also prepared to test the stability of the m/z 245 → 177/m/z 245 → 159 ratio in different concentrations.

QC samples for evaluation of the per cent amounts as well as absolute concentrations of IPP and DMAPP in cellular matrix were prepared by spiking known concentrations of the compounds (0.3 μM, 0.5 μM and 0.7 μM) to untreated MCF-7 cell extracts. A standard of ApppD was not available and therefore similar validation could not be performed for ApppI and ApppD.

2.7. Stability

The stability of the analytes during the analysis process was tested by evaluating the short-term and freeze-thaw stability of the working solutions. IPP and ApppI were diluted with water to final concentration of 1.0 μM and the samples were kept in room temperature for 24 h. Freeze–thaw stability was tested by repeating the freeze and thaw cycle three times before analyzing the content. Post-preparative stability of the samples was tested by resuspending untreated MCF-7 cell samples by vortex mixing to 150 μL of MilliQ-water containing 1.0 μM IPP, ApppI and AppCp, either with or without 0.25 mM NaF and Na_3VO_4 as phosphatase inhibitors. The samples were kept either at room temperature or in a cooled autosampler (4 °C) for maximum of 24 h. The contents of ApppI and IPP were measured at 0 h, 8 h and 24 h after the sample preparation and the degradation percentages were calculated as comparison of the peak areas of the analytes in the sample at 8 h and 24 h to the ones at 0 h.

3. Results and discussion

3.1. Ion-pair HPLC

The highly hydrophilic compounds were separated on a C18 reversed phase column using volatile dimethylhexylamine (DMHA) as an ion-pair agent. The method optimization is presented earlier by our group [29,30]. The MRM chromatograms of the analytes are shown in Fig. 2. The ion-pair modifiers added to the solvents are considered to interfere with the ESI process [43], however, we showed that addition of 2 mM DMHA does not significantly suppress the signal of the analyte [30]. Hence, in the gradient elution of the analytes, the DMHA concentration was decreased from 20 mM to 2 mM with simultaneous increasing of the organic eluent to avoid suppression during the elution of the analytes as well as assist the elution. All compounds including the internal standard eluted at retention times within 55 s and therefore the ionization environment can be expected to be similar for each compound.

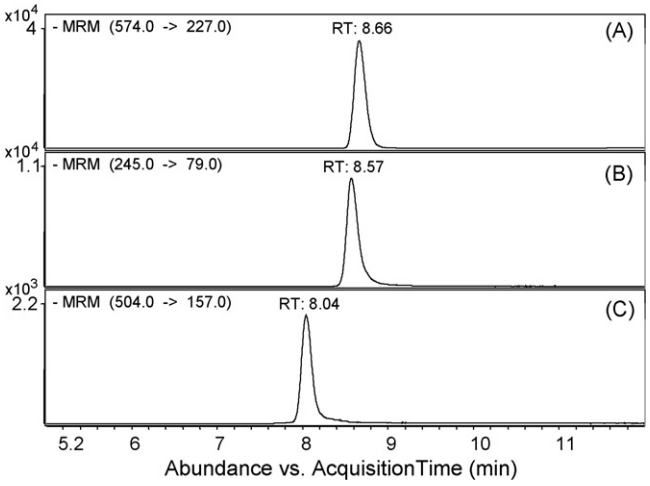


Fig. 2. MRM chromatograms of ApppI/ApppD (A), IPP/DMAPP (B) and ISTD (C).

Table 1
Intra- and inter-day measured concentrations, accuracies and precision for analysis of IPP/DMAPP and ApppI/ApppD.

	IPP						
	conc. (μM)	Measured conc. (μM)		Accuracy (%)		Precision (RSD %)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
QC1	0.045	0.039 ± 0.005	0.042 ± 0.003	88.8	94.0	13.4	6.0
QC2	0.30	0.37 ± 0.02	0.34 ± 0.03	121.7	114.7	6.4	8.4
QC3	0.60	0.68 ± 0.01	0.66 ± 0.08	115.0	101.5	4.6	14.9
QC4	12.0	13.5 ± 0.2	13.0 ± 0.7	113.2	104.5	1.6	9.2
	ApppI						
	conc. (μM)	Measured conc. (μM)		Accuracy (%)		Precision (RSD %)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
QC1	0.030	0.034 ± 0.002	0.032 ± 0.004	114.8	107.7	10.3	13.6
QC2	0.20	0.22 ± 0.01	0.21 ± 0.02	110.3	105.0	7.3	7.5
QC3	0.60	0.65 ± 0.04	0.67 ± 0.06	108.0	111.0	6.2	9.4
QC4	12.0	13.2 ± 0.2	12.7 ± 1.0	110.3	106.0	1.6	7.9

The disadvantage in using ion-pair chromatography is long equilibrium times of the columns, thereby increasing the analysis time. Furthermore, the additives dirty the ion source and columns shortening the column lifetime. These drawbacks of the method could perhaps we avoided by using alternative approaches for the liquid chromatographic separation, such as HILIC.

The isomeric compounds, IPP and DMAPP as well as ApppI and ApppD are structurally similar except for location of the carbon double bond (Fig. 1.). The liquid chromatography method described could not separate these compounds resulting in a coelution of the isomers. However, we were able to determinate the concentration of IPP and DMAPP in the sample by utilizing the signal intensity ratio of characteristic fragment ions in the MS^2 spectra, generated by ion trap instrument. Similarly the amounts of ApppI and ApppD were calculated from fragment ion ratio in the generated MS^3 spectra (see Chapter 3.6).

3.2. Mass spectrometry of IPP/DMAPP, and ApppI/ApppD

Quantitation of the total concentration of the analytes was performed using a triple quadrupole instrument. Deprotonated molecule ions $[M-H]^-$ of the analytes were identified in the full scan spectra at *m*/*z* 245 for IPP/DMAPP and *m*/*z* 574 for ApppI/ApppD. For AppCp molecule ion at *m*/*z* 504 was detected. Each molecule ion was subjected to CID to produce fragment ions and the ions with most intense signal were chosen for the multiple reaction monitoring (MRM). Fragments generated by CID were *m*/*z* 79 for IPP/DMAPP, *m*/*z* 227 for ApppI/ApppD and *m*/*z* 157 for internal standard AppCp (spectrum not shown). The collision energies yielding highest signal intensities for each compound were 15 V, 27 V and 30 V, respectively. Typically fragmentation occurred at the site of the phosphate ester bonds and fragments such as a phosphate group [44] were cleaved. Other fragment ions were also observed when lower collision energies were applied.

3.3. Linearity, accuracy and precision of IPP/DMAPP, and ApppI/ApppD

Limit of quantitation was 0.030 μM for IPP and 0.020 μM for ApppI. Since the range of the method of analyzing the total amount of IPP/DMAPP and ApppI/ApppD was relatively high (2000-fold), the calibration curve was divided into two separate curves both containing five standard points. QC samples were prepared at the low and high point of both curves. Both curves were linear over their calibration range with mean correlation coefficients of 0.998 and 0.996 for lower concentration calibration curve of IPP and ApppI, respectively, and 0.995 and 0.987 for higher concentration calibration curve of IPP and ApppI, respectively. Intra-day accuracy for each QC sample was within 100 ± 21.7% for IPP and 100 ± 14.8% for ApppI. Intra-day precisions were better than 13.4% and 10.3% for IPP and ApppI, respectively. The inter-day results did not differ significantly from the intra-day results. The measured concentrations, accuracies and precisions for each QC sample are displayed in Table 1.

3.4. Extraction recovery and repeatability, matrix effect

The recovery percentage of the extraction method was examined by repeating the acetonitrile–water extraction twice for the same ZOL treated cell sample and comparing the contents. ZOL treated samples were used for the experiments since they contain a significant amount of IPP/DMAPP and ApppI/ApppD, whereas in the untreated control samples these metabolites were not detected. It appeared that relative to the first extraction, only approximately 5% of the total amount was covered during the second extraction. Thus, it was concluded that the extraction using acetonitrile–water was sufficient to extract the majority of the analytes from the cell culture samples with relatively low amount of interferences. Also, it simultaneously precipitates the proteins that can be easily separated for the protein concentration measurement. The repeatability of the extraction method, expressed as percent relative standard deviation (RSD %), calculated from the peak area ratios of the MRM chromatograms of the analytes and internal standard were 5.9% and 6.2% for IPP/DMAPP and ApppI/ApppD, respectively. Matrix induced ion suppression was not significant for IPP and ApppI standards.

3.5. Stability

Working solutions of IPP and ApppI were stabile at room temperature for at least 24 h (accuracies 105.7% and 107.9%, respectively) and after three freeze–thaw cycles (accuracies 102.2% and 104.1%, respectively).

Post-preparative stability of the samples was tested by storing the samples at four different conditions: (1) at room temperature without phosphatase inhibitors, (2) at room temperature with 0.25 mM phosphatase inhibitors (NaF and Na_3VO_4), (3) in a cooled autosampler at 4 °C without phosphatase inhibitors, and (4) in a cooled autosampler at 4 °C with 0.25 mM phosphatase inhibitors. The per cent amounts of the analytes compared to the samples at 0 h after 8 h and 24 h storage are shown in Fig. 3. Significant degradation of the analytes occurred without cooling and without presence of the phosphatase inhibitors after 8 h, and after 24 h only less than 20% of IPP and 3% of ApppI compared to the initial amount was detected. It was concluded that ApppI is stable for at least 24 h at

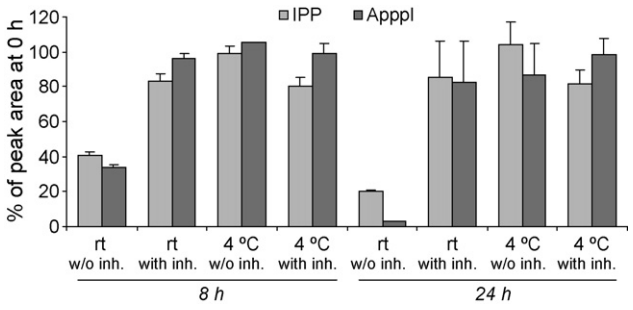


Fig. 3. Post-preparative stability of IPP and ApppI standards spiked in MCF-7 cell extract, presented as percentage of the peak area of the analyte at 0 h.

4 °C with 0.25 mM phosphatase inhibitors (98.2% recovery) and that in similar conditions the recovery for IPP was 81.5%. No significant degradation of AppCp was found after 24 h storage in any conditions (data not shown).

3.6. Relative amount of the isomeric compounds

The isomers IPP and DMAPP could not be separated chromatographically during this study. However, the concentration of both of the compounds in the sample was measured utilizing differences in their MS^2 spectra. The fragment ion at m/z 177, formed by cleavage of pyrophosphate group [44,45], was more intense in the MS^2 spectrum of IPP than in that of DMAPP (Fig. 4.). Presumably, the cleavage of the pyrophosphate group of DMAPP would result in a formation of an unstable fragment (Fig. 4. B) and thus the formation of fragment ion m/z 177 is not as favorable for DMAPP as it is for IPP (Fig. 4. A). Other fragments ions at m/z 79, m/z 159 and m/z 227 were also seen in the negative MS^2 spectra of IPP and DMAPP, possibly corresponding to a phosphate group [44], a pyrophosphate moiety [34,44], and a cleavage of a water molecule, respectively. The proposed formations of the fragment ions are described in Scheme 1. The MS^2 spectrum of internal standard AppCp showed two fragment ions, corresponding to $[M-H_3PO_4]^-$ and $[M-H_2O]^-$, with high signal intensity. Transition m/z 504 → 406 was selected for the MRM method (the spectrum not shown). The MS^2 spectra produced by a triple quadrupole instrument did not distinguish between the isomers and generally the only fragment ion produced by CID was m/z 79.

The relative amount of IPP and DMAPP isomers were calculated using following strategy; a calibration curve was generated, where the peak area ratio of the MRM chromatograms m/z 245 → 177 and m/z 245 → 159 was plotted against the relative amount of IPP and DMAPP (Fig. 5. A). The percentage of IPP of the total amount in the sample was calculated using this calibration curve, and the percentage of DMAPP was expected to be 100%—% of IPP. The absolute concentration of IPP was calculated using calibration curve prepared by spiking IPP standard in untreated cell lysates over a range of 0.045–15.0 μM. The samples also contained 1.0 μM AppCp. The curve was generated by plotting the MRM peak area ratio of m/z 245 → 159 and ISTD against the concentration. It was found out, however, that the signal intensity of m/z 245 → 159 was 35.5% lower for pure standard of DMAPP to that of IPP. Therefore, the peak area of IPP had to be corrected in order to evaluate the absolute concentration of IPP in the sample. A corrected peak area of IPP was calculated using the equation (Fig. 5. B). Peak area of m/z 245 → 159 was obtained from MRM chromatogram, relative amount of IPP and DMAPP were calculated using the calibration curve (Fig. 5.) and factor 0.6446 was obtained as a signal intensity ratio of m/z 245 → 177 and m/z 245 → 159 of pure standards of DMAPP and IPP,

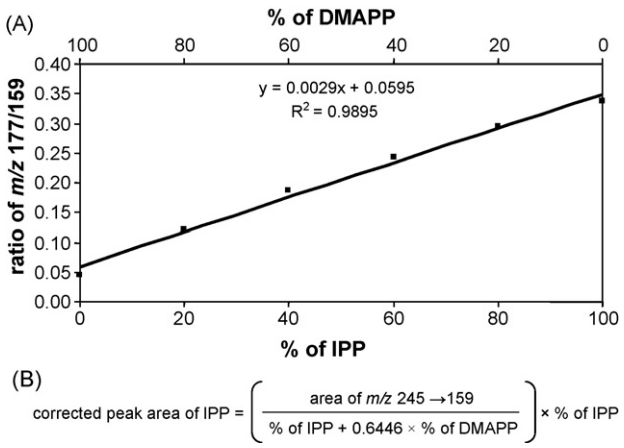


Fig. 5. A strategy to calculate the relative amount of IPP and DMAPP isomers: peak area ratio of MRM chromatograms m/z 245 → 177 and m/z 245 → 159 is plotted against the per cent amount of IPP and DMAPP to create a calibration curve to calculate percentual amount of IPP. Since not all the signal m/z 245 → 159 is coming from IPP, the MRM chromatogram peak area (m/z 245 → 159) is corrected using the equation due to lower signal intensity of m/z 245 → 159 of DMAPP compared to IPP. Factor 0.6446 is the response factor of pure DMAPP and IPP standards. The absolute concentration of IPP is calculated utilizing the standard curve prepared by spiking IPP in untreated cell lysates, the corrected peak area and the per cent amount of IPP. Concentration of DMAPP is calculated similarly and the per cent amount was calculated by subtracting the per cent amount of IPP from the total amount.

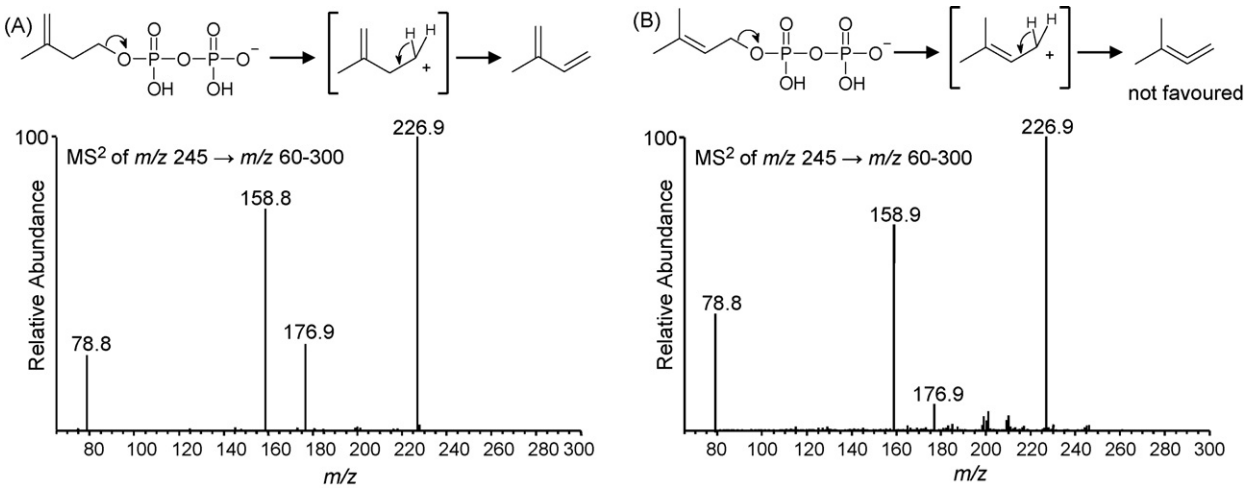
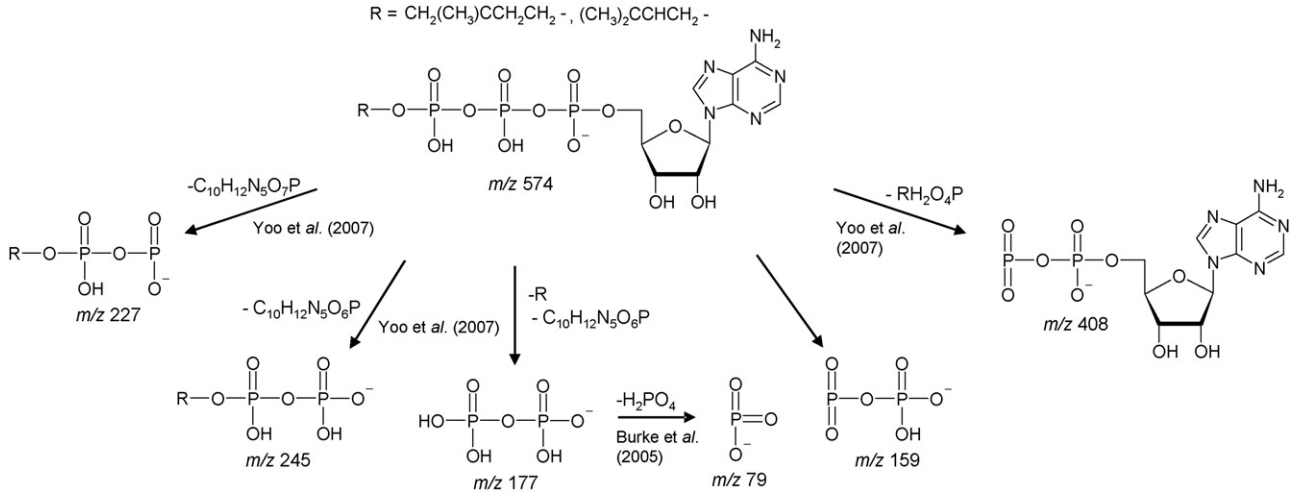


Fig. 4. Suggested fragmentation mechanism and negative MS^2 spectra of IPP (A) and DMAPP (B), obtained by ion trap instrument.



Scheme 1. Proposed fragmentations of ApppI and ApppD.

respectively. The corrected peak area of IPP was compared to the calibration curve prepared in cell extract to obtain the concentration of IPP in the cell sample. The absolute concentration of DMAPP in the same sample was calculated utilizing the relative amount of DMAPP and the absolute concentration of IPP.

The curve slope of the calibration curve with varying amounts of IPP and DMAPP was 0.0029 and R^2 0.990. Accuracies of three QC samples ($n = 6$) were within $100 \pm 13.8\%$ and precisions better than 20.1%. Exact values are presented in Table 2A. The signal intensity ratio of m/z 245 → 177 and m/z 245 → 159 remained constant in all three concentrations tested, with average value of 0.218 and RSD 2.4%, indicating as expected, that the ratio does not vary with the concentration. The QC samples prepared in cell extracts showed accuracies within $100 \pm 19.1\%$ for per cent amounts and within $100 \pm 16.6\%$ for absolute concentrations. Precisions were better than 10.4% for per cent amounts and 13.1% for absolute concentrations, respectively. The exact values are displayed in Table 2B.

Attachment of IPP/DMAPP moiety to AMP in the ZOL treated cells has been shown in our previous studies. In this study MS^3 spectra were used to estimate the ratio of IPP and DMAPP in the ATP conjugate. The conjugate shows a fragment ion at m/z 245 in the MS^2 spectrum produced by cleavage of an adenosine monophosphate moiety (Fig. 6. A). The method is based on presumption that the MS^3 fragmentation of ion m/z 574 → 245 is similar to the MS^2 fragmentation of IPP and DMAPP ions. This was justified by the observed MS^2 and MS^3 spectra, which showed same fragments in MS^2 spectra of IPP and DMAPP (Fig. 4.) as well as in MS^3 spectrum of ApppI (Fig. 6. B). Therefore, it was possible to calculate the relative amounts of ApppI and ApppD in the cell culture sample using the response factors of m/z 245 → 177 and m/z 245 → 159 of IPP and DMAPP and a calibration curve generated by spiking ApppI standard in non-treated MCF-7 cell lysates. The presence of ApppD in the ZOL treated cancer cell samples was showed for the first time in this study.

3.7. MCF-7 cell samples

We have studied the mevalonate pathway metabolite accumulation and ApppI/ApppD accumulation after N-BP treatment in various publications [19,20,23]. We have shown that MCF-7 cells produce a significant amount of IPP/DMAPP and ApppI/ApppD compared to other cell lines and that treatment with 25 μM ZOL for 24 h sufficiently inhibits the FPP synthase [20]. Therefore, the same cell line and treatment conditions were chosen to demonstrate the

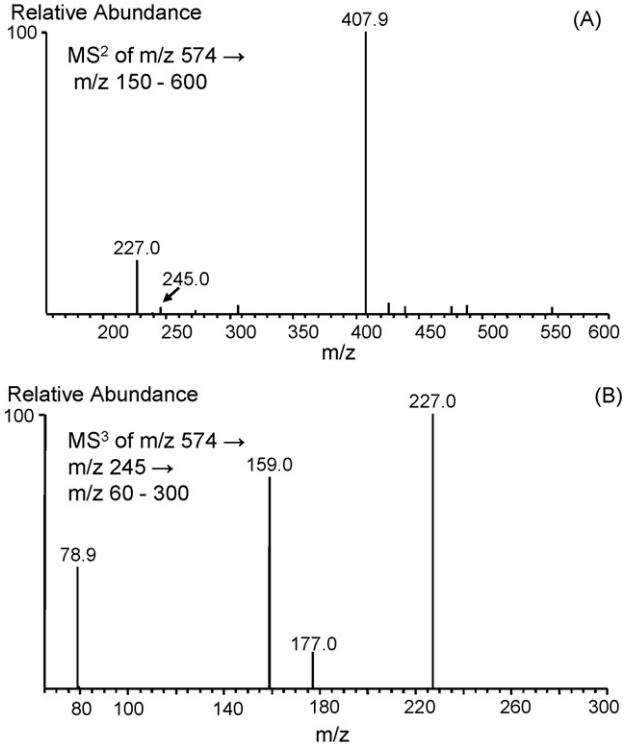


Fig. 6. Negative MS^2 (A) and MS^3 (B) spectra of ApppI/ApppD in the ZOL treated MCF-7 cell sample.

effectiveness of the method to extract and detect the metabolites for cell culture samples.

Similar amount of cells were used for each sample and the molar amounts (nmol) of the analytes were also normalized to on milligram (mg) of protein. In the ZOL treated MCF-7 samples ($n = 6$), the amount of IPP/DMAPP was 2.4 nmol/mg protein and the amount of ApppI/ApppD was 1.1 nmol/mg protein. These results are in correlation with previous studies [23]. In the untreated control cell samples ($n = 6$) IPP/DMAPP and ApppI/ApppD were not detected. The relative amounts of IPP and DMAPP in the cancer cell culture samples were $16.2 \pm 1.2\%$ and $83.8 \pm 1.2\%$, respectively, whereas the amounts of ApppI and ApppD were $30.0 \pm 10.2\%$ and $70.0 \pm 10.2\%$, respectively. Consequently, we found the ratio of IPP:DMAPP is 1:4. Approximate ratio of ApppI:ApppD was 3:7, however, due to lower signal intensity there was more variability between the samples

Table 2A
Calculated percentages, accuracies and precisions for IPP and DMAPP.

	Percentual amount (%)		Calculated amount (%)		Accuracy (%)		Precision (RSD %)	
	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP
QC 1	10	90	10.0 ± 2.0	90.8 ± 2.7	100.2	100.0	20.1	3.0
QC 2	50	50	56.0 ± 3.7	43.7 ± 3.6	112.6	87.4	6.5	8.4
QC 3	90	10	94.1 ± 4.4	8.6 ± 1.6	104.5	86.2	4.7	18.3

$n = 6$.

Table 2B
Accuracy and RSD values of the concentration and percentual values of IPP and DMAPP in spiked MCF-7 cell extracts.

	Percentual amount (%)		Calculated percentual amount (%)		Accuracy (%)		Precision (RSD %)	
	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP
QC1	30	70	32.5 ± 3.4	67.0 ± 4.7	108.4	95.8	10.4	7.0
QC2	50	50	46.8 ± 2.6	51.9 ± 1.5	93.6	103.7	5.5	2.9
QC3	70	30	64.3 ± 2.4	35.7 ± 3.3	91.8	119.1	3.7	9.3
	Concentration (uM)		Calculated concentration (uM)		Accuracy (%)		Precision (RSD %)	
	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP	IPP	DMAPP
QC1	0.3	0.7	0.35 ± 0.03	0.72 ± 0.09	116.6	102.3	8.0	13.1
QC2	0.5	0.5	0.46 ± 0.04	0.50 ± 0.01	92.6	99.5	8.2	2.1
QC3	0.7	0.3	0.60 ± 0.04	0.33 ± 0.03	85.9	111.2	5.8	8.7

compared to IPP and DMAPP. Regardless of that, there was a clear correlation between the IPP:DMAPP and ApppI:ApppD ratio, concluding that majority of the accumulated MVA pathway metabolite and corresponding ATP conjugate consists of DMAPP.

4. Conclusion

A method for analysis of IPP, DMAPP and ATP analogs ApppI and ApppD in the ZOL treated MCF-7 cell culture samples was developed. Determination of the isomeric compounds in cells was based on measurement of MS^2 and MS^3 spectra signal intensity ratio of two characteristic fragment ions. We found out that the ratio of IPP and DMAPP in the ZOL treated MCF-7 cells is approximately 1:4. In addition, the ratio of ATP analogs ApppI and ApppD was approximately 3:7, showing distinct correlation to the ratio of IPP:DMAPP. There is no previous data about the relative amounts of these compounds and our method provided novel information about the composition of these compounds in cancer cells. The method enables analysis of these metabolites after simple and fast sample preparation from cell cultures. The ion trap instrument shows remarkably different fragment ions compared to triple quadrupole instrument, and was used to separate quantitation of each isomer. The triple quadrupole instrument was used for analysis of IPP/DMAPP and ApppI/ApppD mixtures. Both methods provided sufficient sensitivity, selectivity and linearity range. The analysis method of IPP/DMAPP and ApppI/ApppD has been successfully applied by our group to studies on intracellular accumulation of the metabolites in various samples [19–23].

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