

Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human $\gamma\delta$ T cells in *Escherichia coli*

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Received 18 September 2001; revised 26 October 2001; accepted 26 October 2001

First published online 22 November 2001

Edited by Thomas L. James

Abstract The *gcpE* and *lytB* gene products control the terminal steps of isoprenoid biosynthesis via the 2-C-methyl-D-erythritol 4-phosphate pathway in *Escherichia coli*. In *lytB*-deficient mutants, a highly immunogenic compound accumulates significantly, compared to wild-type *E. coli*, but is apparently absent in *gcpE*-deficient mutants. Here, this compound was purified from *E. coli* Δ *lytB* mutants by preparative anion exchange chromatography, and identified by mass spectrometry, ¹H, ¹³C and ³¹P NMR spectroscopy, and NOESY analysis as (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP). HMB-PP is 10⁴ times more potent in activating human V γ 9/V δ 2 T cells than isopentenyl pyrophosphate. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: $\gamma\delta$ T cell; 2-C-Methyl-D-erythritol 4-phosphate pathway; 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; *gcpE*; *lytB*

1. Introduction

$\gamma\delta$ T cells constitute approx. 0.5–5% of human peripheral blood T cells, the majority of which express the V γ 9/V δ 2 T cell receptor (TCR) [1]. Unlike conventional MHC-restricted CD4⁺ or CD8⁺ T cells expressing the $\alpha\beta$ TCR, V γ 9/V δ 2 T cells are activated by small non-peptide phosphoantigens, such as isopentenyl pyrophosphate (IPP) [2–5]. While IPP was the first ligand described for the human V γ 9/V δ 2 TCR, we recently demonstrated that this weak agonist does not represent the natural ligand but rather a much more potent IPP precursor derived from the 2-C-methyl-D-erythritol 4-

phosphate (MEP) pathway of isoprenoid biosynthesis [6,7]. This alternative pathway is utilized by algae and plastids of higher plants, as well as by many pathogenic bacteria and apicomplexan protozoa [8,9], whereas isoprenoid biosynthesis in eukaryotes, archaeobacteria, and some eubacteria occurs via the classical mevalonate pathway [9,10]. Thus, this unusual recognition of common metabolites explains the ability of V γ 9/V δ 2 T cells to react towards a large number of infectious agents, such as *Francisella*, *Mycobacterium*, *Pseudomonas*, and *Salmonella*, as well as to malaria parasites [11,12].

In order to differentiate the terminal enzymatic steps of the MEP pathway [9,13,14], we have constructed *Escherichia coli* mutant strains deficient in the *gcpE* and *lytB* gene, respectively [15,16]. Both genes control enzymatic reactions leading from the last clearly identified intermediate of the MEP pathway, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate (MEcPP), to IPP [15–18], with the V γ 9/V δ 2 T cell-activating compound as a putative intermediate in the reaction sequence. Interestingly, low molecular weight extracts prepared from Δ *gcpE* mutants have a significantly reduced capacity to stimulate human V γ 9/V δ 2 T cells, compared to wild-type *E. coli*, arguing for a role of the GcpE enzyme in the biosynthesis of the $\gamma\delta$ T cell activator [7]. In striking contrast, in Δ *lytB* bacteria the V γ 9/V δ 2 T cell-activating compound accumulates approx. 150-fold over the level in wild-type *E. coli*; electrospray ionization mass spectrometry (ESI-MS) analysis showed that this molecule is a pyrophosphorylated compound with a mass of 262 Da². In the present study, the V γ 9/V δ 2 T cell activator was purified from *E. coli* Δ *lytB* mutants by preparative anion exchange chromatography, and the chemical structure was resolved by NMR spectroscopy as (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.

2. Materials and methods

2.1. Purification of the $\gamma\delta$ T cell activator

The *E. coli* wt Δ *lytB* mutant with a precise in-frame deletion of the *lytB* gene deriving from the *E. coli* K-12 strain ATCC 23716 was constructed using the pKO3 vector as described previously [16]. A liquid culture of *E. coli* wt Δ *lytB* mutant was grown in a 1 l bioreactor (Braun Biotech, Melsungen, Germany) in minimal medium supple-

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Abbreviations: DOXP, 1-deoxy-D-xylulose 5-phosphate; ESI, electrospray ionization; FBPP, 3-formyl-1-butyl pyrophosphate; HMBC, heteronuclear multiple bond connectivity; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMQC, heteronuclear multi-quantum coherence; IPP, isopentenyl pyrophosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TCR, T cell receptor

² Eberl, M., Altincicek, B., Kollas, A.K., Sanderbrand, S., Bahr, U., Reichenberg, A., Beck, E., Wiesner, J., Hintz, M., and Jomaa, H., Accumulation of a potent $\gamma\delta$ T cell stimulator after deletion of the *lytB* gene in *Escherichia coli*, submitted for publication.

mented with 25 g/l glucose, 25 mg/l ampicillin (Sigma, Taufkirchen, Germany) and 400 μ M mevalonate (Fluka, Taufkirchen, Germany) to an optical density of 9.5 at 600 nm. Bacteria cells were pelleted at $17000\times g$ for 15 min, resuspended in 100 ml of 50 mM ammonium formate pH 8.0 (Fluka), and ruptured using an EmulsiFlex-C5 homogenizer (Avestin, Heidelberg, Germany) at a pressure of 150 MPa. The soluble fraction of the homogenate was passed twice over Isolute C18 reversed phase columns (International Sorbent Technology, Mid Glamorgan, UK). Subsequently, the low molecular weight fraction of the flow-through was collected by cross-flow diafiltration on a hollow fiber membrane with a 3 kDa cut-off (A/G Technology, Needham, MA, USA), at a trans-membrane pressure of 130 MPa and a permeate volume current of $39.5 \text{ l h}^{-1} \text{ m}^{-1} \text{ MPa}^{-1}$. Anion exchange chromatography was performed on a Source 15Q column (Amersham Pharmacia, Freiburg, Germany) with a bed volume of 100 ml, using a Pharmacia FPLC device. Buffers used were: A, 26.3 mM ammonium formate pH 8.0; B, 500 mM ammonium formate pH 8.0; C, 1 M ammonium formate pH 8.0; and chromatography was done with a flow rate of 10 ml/min as follows: wash, 70 min 95% A, 5% B; elution, linear gradient from 95% A, 5% B to 100% B in 126 min; 20 min 100% B; 30 min 100% C; equilibration, 50 min 95% A, 5% B. Absorbance was detected at 280 nm, and fractions were collected every 30 s, corresponding to a volume of 5 ml each.

2.2. Phosphate determination

For measurement of organically bound phosphate, a method developed for HPLC detection was modified [19]. Speedvac-dried aliquots of 200–300 μ l were redissolved in 100 μ l 4% ammonium peroxodisulfate (Fluka) and boiled for 1 h. Subsequently, the samples were mixed with 50 μ l H₂O and 600 μ l 63.5 mM (NH₄)₂Mo₂O₇ (Fluka), 6.4 mM NH₄VO₃ (Fluka), 6.8% HNO₃ (Roth), and incubated at room temperature for 30 min. Absorbance was measured at 410 nm against water on a U-2000 spectral photometer (Hitachi, San Jose, CA, USA), and calibrated using a standard curve of serial dilutions of 3 mM Na(NH₄)HPO₄ (Sigma) treated in the same way. Different dilutions of a 2 mM stock of ethylphosphonic acid (Lancaster, Morecambe, UK) were used as standard and gave a recovery of $98 \pm 5\%$ (data not shown); cytidine diphosphate (Sigma) and farnesyl pyrophosphate (Sigma) used as controls yielded a phosphate recovery of 80–90% (data not shown).

2.3. Bioactivity assays

Flow cytometric analysis of human V γ 9/V δ 2 T cells was performed as described recently². In brief, 2×10^5 peripheral blood mononuclear cells were seeded in 200 μ l RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 25 μ g/ml gentamicin, 10 U/ml human interleukin-2 (all from Life Technologies, Karlsruhe, Germany), and 10% human AB serum (Bayerisches Rotes Kreuz, Augsburg, Germany). Bacterial samples were tested at appropriate dilutions and calibrated using a standard curve of serial dilutions of IPP (Sigma). Cells were analyzed 18 h later on an Epics XL flow cytometer supported with Expo32 software (Beckman Coulter, Krefeld, Germany), using CD3-PC5 (UCHT1), V γ 9-FITC (Immu360) and CD69-PE (TP1.55.3) monoclonal antibodies (Beckman Coulter). Bioactivity of bacterial extracts was determined from three independently analyzed blood donors, and expressed as the stimulatory capacity comparable to an equivalent concentration of IPP.

2.4. Mass spectrometry

Negative mode ESI-MS was done on a Micromass ZQ 4000 (Waters), with a transfer capillary temperature of 150°C. Samples were diluted 1:5 in methanol and analyzed by single injection at 20 μ l/min. Mass peaks were integrated from 50 scans accumulated over 1 min. Accurate mass analysis for determination of elemental composition was performed using an ESI orthogonal-time-of-flight MS (Mariner; PE Biosystems, Framingham, MA, USA) with internal calibration, equipped with a nano-ESI source (Protana, Odense, Denmark). 3 μ l of sample solutions were filled in gold-coated nanospray capillaries and sprayed at flow rates of 10–30 nl/min. The voltage of the nanospray needle was set to 700 V; the temperature of the heated transfer capillary was 200°C. Four single mass determinations obtained from accumulation of 50 scans over 10 s each were averaged. Structural analysis by MS-MS of selected ions was performed using a quadrupole ion trap mass spectrometer (LCQ; Thermo Finnigan MAT, Bremen, Germany) with a relative collision energy between

20 and 30%. Spectra were taken in the negative ion mode and averaged over 10 scans each.

2.5. NMR spectroscopy

Analysis by NMR spectroscopy was done on the Δ lytB anion exchange fraction containing the peak bioactivity and showing the most prominent mass signal at m/z 261, as well as on the neighboring fractions as control. The fractions were precipitated overnight at 4°C in the presence of 100 mM BaCl₂ and 80% ethanol, washed with 80% ethanol, dried in vacuum, and redissolved in an appropriate volume of D₂O (Sigma). Spectra from ¹H NMR, heteronuclear correlation analysis (HMQC) and long-range correlation spectroscopy (HMBC) were recorded in D₂O at 400 MHz using a DRX 400 spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5 mm broad band triple resonance gradient probe. Experimental frequencies were 400.13 MHz and 100.62 MHz for ¹H and ¹³C, respectively. All measurements were carried out at 300 K and referenced to external tetramethylsilane in CDCl₃. Parameters of the gs-HMQC and gs-HMBC experiments were: spectral widths in the proton dimensions, 5.6 ppm, in the carbon dimensions, 210 ppm; 1024 points in the f_2 dimension, and 512 increments in the f_1 dimension; 64 scans for each increment and an experimental time of 20 h for the gs-HMQC, and 128 scans for each increment and an experimental time of 44 h for the gs-HMBC. Parameters of the gs-NOESY experiment with WATERGATE [20,21] for solvent suppression were: spectral widths in the proton dimensions, 10.0 ppm; 8192 points in the f_2 dimension, and 256 increments in the f_1 dimension; 64 scans for each increment; mixing time $\tau_m = 500$ ms; experimental time 13 h. As reference compounds, (*E*)-4-[(*tert*-butyldimethylsilyloxy)-3-methyl-2-buten-1-yl]butanoate [22], (*Z*)-4-[(*tert*-butyldimethylsilyloxy)-3-methyl-2-buten-1-yl]butanoate [23] and (*E*)-1-[(tetrahydro-2H-pyran-2-yl)oxy]-3-methyl-2-buten-1-ol [24] were synthesized (data not shown), and used for gs-NOESY experiments with the following parameters: spectral widths in the proton dimensions, 10.0 ppm; 1024 points in the f_2 dimension, and 128 increments in the f_1 dimension; 16 scans for each increment; mixing time $\tau_m = 500$ ms; experimental time 1.5 h. Data were processed with the software package X-WINNMR (Bruker) after apodization with a sine bell weighting in f_1 and f_2 (gs-HMQC and gs-NOESY: SSB=2; gs-HMBC: SSB=0). Quadrature detection in the indirect dimension was achieved by gradient selection (gs-HMQC and gs-HMBC), or States-TPPI (gs-NOESY) [25]. NOESY spectra for the (*E*)-reference compounds were referenced to tetramethylsilane (0.0 ppm), for the (*Z*)-reference compound to CDCl₃ (7.24 ppm). Proton-decoupled ³¹P NMR spectra were recorded at 161.975 MHz and referenced to external phosphoric acid (0.0 ppm). Chemical shifts were determined with an accuracy of ± 0.05 ppm for ¹³C and ³¹P NMR spectra, and ± 0.005 ppm for ¹H NMR spectra; coupling constants were determined with an accuracy of ± 0.1 Hz.

3. Results and discussion

A 3 kDa ultrafiltrate was prepared from a 1 l culture of *E. coli* wt Δ lytB mutants, and fractionated by anion exchange chromatography. The bioactivity of all fractions was measured as the capacity for up-regulating the CD69 activation marker on the surface of V γ 9/V δ 2 T cells, and expressed as IPP equivalent (Fig. 1B). The peak bioactivity correlated well with the occurrence of an m/z 261 ion in the negative ion mode $[M-H]^-$ and the presence of a high phosphate content (Fig. 1C,D).

Negative mode MS-MS analysis of the deprotonated molecule at m/z 261 yielded a loss of water (m/z 243), as well as peaks corresponding to phosphate and pyrophosphate ions, m/z 97 H₂PO₄⁻ and m/z 177 H₃P₂O₇⁻, together with the corresponding dehydrated ions, m/z 79 PO₃⁻ and m/z 159 HP₂O₆⁻; a signal at m/z 163 indicated a loss of a single phosphoric acid moiety, H₃PO₄ (Fig. 2). MS-MS-MS analysis of the m/z 243 ion showed a mass at m/z 225, indicating the presence of a further OH-group in this molecule (data not shown). Accurate mass determination of m/z 261 gave a

monoisotopic mass of 260.9937 ± 0.0007 Da. With a general formula $C_{1-20}H_nO_{7-10}N_{0-5}P_{2-5}$ (containing P_2O_7 for the pyrophosphate group) and a mass accuracy of ± 0.001 Da, only one elemental composition matched with the determined mass: $C_5H_{12}O_8P_2$ for the pyrophosphorylated metabolite.

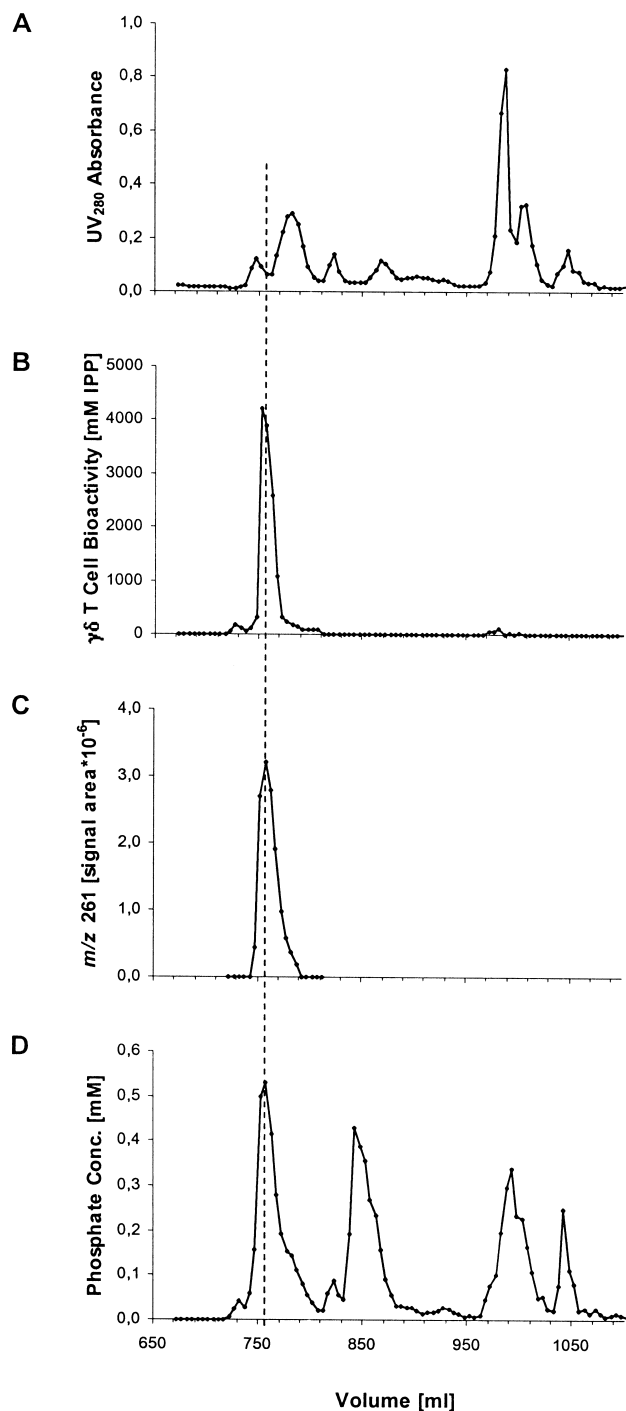


Fig. 1. Analysis of fractions with $\gamma\delta$ T cell bioactivity obtained by anion exchange chromatography. A: UV absorbance at 280 nm. B: $\gamma\delta$ T cell bioactivity in IPP equivalents (averaged from three blood donors). C: Mass spectrometry detection of the m/z 261 ion, expressed as the integrated area of the mass signal. D: Phosphate concentration after oxidative release of organically bound phosphate groups.

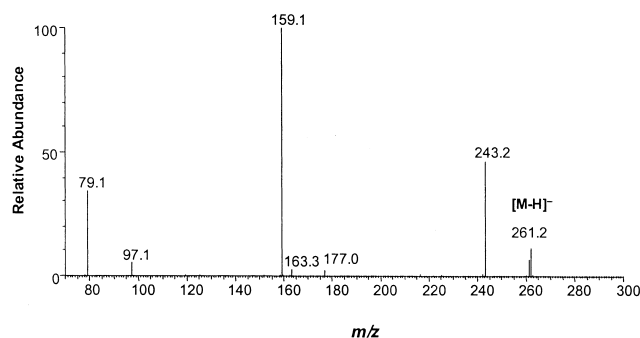


Fig. 2. Ion trap ESI-MS of the m/z 261 ion. MS-MS spectra were taken in the negative ion mode and averaged over 10 scans each.

1H NMR spectroscopy data were compared to known NMR spectra of related molecules [26–32], and led to the proposed structure of 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (Fig. 3). A singlet at $\delta=1.62$ ppm could be assigned to the methyl group bound to C3 (s, 3H, CH_3), and a singlet at $\delta=3.93$ ppm to the methylene protons bound to C4 (s, 2H, CH_2OH). A doublet of doublets at $\delta=4.44$ ppm belonged to the methylene protons bound to C1 (dd – appearing as pseudo-triplet, 2H, $J_{H-H} = J_{H-P} = 6.9$ Hz, CH_2OP) and a triplet at $\delta=5.57$ ppm originated from the olefinic proton on C2 (t, 1H, $J=6.5$ Hz, $HC=C$). The HDO signal was at 4.64 ppm. ^{13}C signals were obtained from 1H - ^{13}C HMQC and HMBC experiments, and the chemical shifts observed, at $\delta=13.1$ (CH_3), 62.3 (CH_2OP), 66.7 (CH_2OH), 120.8 ($HC=C$), 139.8 ($HC=C$), were consistent with the proposed structure and verified the connections of the carbon scaffold (Fig. 3). Any additional signals visible could be related to contamination with ammonium formate and ethanol. Finally, ^{31}P NMR spectroscopy confirmed the presence of a pyrophosphate group; the chemical shifts observed were at $\delta=-4.56$ (d, $J=19.6$ Hz) and -4.86 (d, $J=21.1$ Hz). This small difference in the couple constants most likely reflects residual 1H coupling, leading to an imprecise value for the P atom connected to the C-scaffold. Importantly, our data from HMB-PP were in remarkable accordance with the NMR spectra of the corresponding alcohol, ‘4-hydroxy-3-methyl-but-2-en-1-ol’ (=2-methyl but-2-ene-1,4-diol): δ_H (200 MHz, $CDCl_3$) 1.65 (s, 3H, CH_3), 3.23 (br, s, 2H, OH), 3.97 (s, 2H, CH_2OH), 4.16 (d, 2H, $J=6.8$ Hz, CH_2OH), 5.61 (tq, 1H, $J=6.8$ Hz and 1.4 Hz, $HC=C$); δ_C (50 MHz, $CDCl_3$) 13.63 (CH_3), 58.69 (CH_2OH), 67.38 (CH_2OH), 123.26 ($HC=C$), and 137.99 ($HC=C$) [28].

In principle, two stereoisomers of HMB-PP are possible, with the CH_2OH group in the *E* or *Z* orientation at the double bond, with respect to the CH_2OPP group. Resolution of the stereochemistry at the double bond was achieved by NOESY experiments, using HMB-PP and closely related reference compounds described previously [22–24]. Thus, in (*Z*)-4-[(*tert*-butyldimethylsilyloxy)-3-methyl-2-butenate, NOE contacts could be detected between the olefinic proton H-2 and the methylene protons H-5 but not between H-2 and the methylene protons H-4 (Fig. 4, left panel). In contrast for the corresponding (*E*)-stereoisomer, NOE contacts were between H-2 and H-4 but not between H-2 and H-5 (Fig. 4, center panel). Similar contacts were observed between H-2 and H-4 of (*E*)-1-[(tetrahydro-2H-pyran-2-yl)oxy]-3-methyl-2-buten-4-ol (data not shown). Importantly, the NOESY spectra of

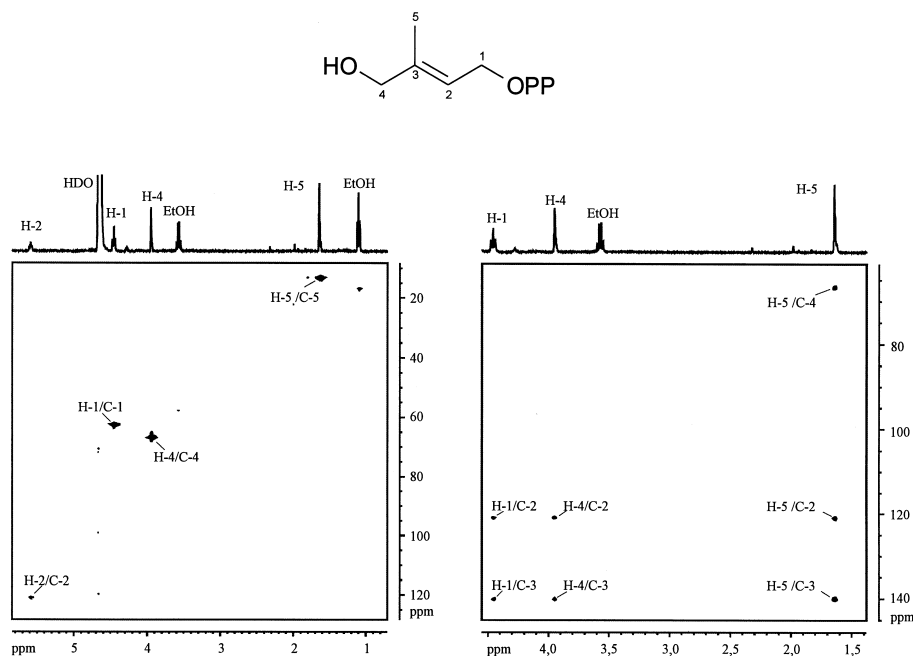


Fig. 3. NMR spectra of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate. Assignment of ^1H and ^{13}C signals by HMQC analysis (left panel), and determination of quaternary carbon and long-range couplings by HMBC analysis (right panel). The ^1H and ^{13}C chemical shifts were referenced to tetramethylsilane (0.0 ppm) as external standard. Horizontal axis, ^1H shifts; vertical axis, ^{13}C shifts.

HMB-PP showed contacts comparable to the (*E*)-reference compounds, indicating proximity of the olefinic proton H-2 and the methylene protons H-4. Consequently, the chemical structure of the $V\gamma 9/V\delta 2$ T cell activator could be established as (*E*)-HMB-PP.

Given a pyrophosphorylated nature of the $V\gamma 9/V\delta 2$ T cell activator, its maximum concentration in the peak fractions can be calculated as approx. 250 μM (= 65 $\mu\text{g}/\text{ml}$). As these fractions exhibited a $V\gamma 9/V\delta 2$ T cell bioactivity equivalent to approx. 4.2 ± 1.1 M IPP (= 1.0 ± 0.26 mg/ml), it can be estimated that this compound is between 12 500 and 21 500 times more active than IPP. This value is considerably higher than for any other natural metabolite isolated so far, and in the range of the most potent synthetic $V\gamma 9/V\delta 2$ T cell activator known, iodohydrin pyrophosphate [4].

Recently, a mycobacterial metabolite stimulating proliferation of $V\gamma 9/V\delta 2$ T cells was described as 3-formyl-1-butyl pyrophosphate (FBPP) [30,31], and subsequently isolated from *E. coli* [33]; because of its structural similarity with IPP, FBPP was suggested a putative novel intermediate of the MEP pathway [31]. However, the molecular structure of FBPP was only indirectly deduced from MS-MS analysis and FBPP, HMB-PP, as well as other pyrophosphorylated compounds such as EpoxPP yield identical fragmentation patterns ([31,33,34]; Fig. 2). Whereas in the present study the chemical structure of HMB-PP could be unambiguously resolved by HMQC, HMBC and NOESY analysis, the nature of FBPP has to remain speculative as the NMR data available [31] differ from those of comparable compounds [26,32]. Although there is only little evidence that HMB-PP is a true intermediate of the MEP pathway and not merely a side-product, the chemical structure of HMB-PP is in accordance with incorporation studies using deuterium-labelled ME isotopomers [35], and insinuates a very elegant mechanism for the still unclear biosynthetic reaction sequence from MEcPP to IPP (Fig. 5).

Nevertheless, it is thinkable that besides GcpE and LytB there may still be one or several more gene products involved in the MEP pathway.

Taken together, we have identified HMB-PP as a major $V\gamma 9/V\delta 2$ T cell activator in *E. coli*. This compound is derived

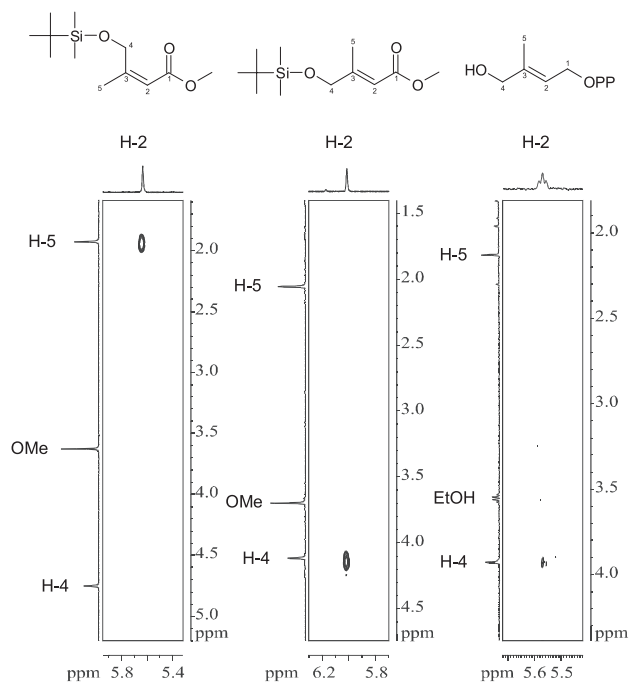


Fig. 4. Characterization of the stereochemistry by NOESY analysis. Assignment of NOE contacts between the methylene protons H-4 and the methyl protons H-5, and the olefinic proton H-2 in (*Z*)-HMB-PP (right panel), in comparison with two closely related compounds in (*Z*)-configuration (left panel) and (*E*)-configuration (center panel). OMe, protons of the methyl ester residue.

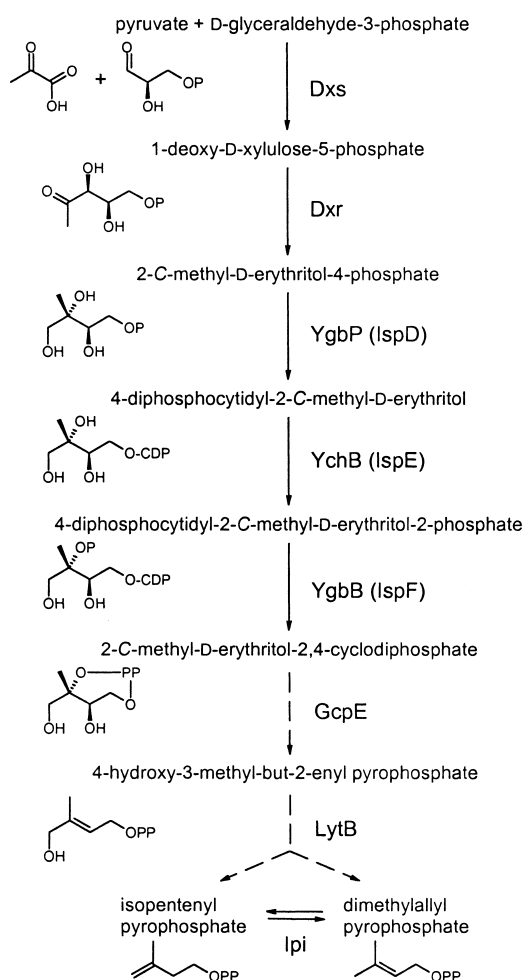


Fig. 5. Proposed biosynthetic reaction steps of the MEP pathway in *E. coli*. Initially, 1-deoxy-D-xylulose 5-phosphate (DOXP) is converted into MEP by DOXP reductoisomerase (Dxr). Subsequently, the enzymes YgbP, YchB and YgbB catalyze the transformation of MEP into MEcPP. GcpE is involved in the next step(s) using MEcPP as substrate to produce the $\gamma\delta$ T cell activator, HMB-PP, whereas LytB mediates the subsequent reaction leading to IPP and dimethylallyl pyrophosphate.

from the MEP pathway of isoprenoid biosynthesis, as it accumulates after deletion of the *lytB* gene and is lacking after deletion of the *gcpE* gene. Moreover, docking analysis shows that HMB-PP fits readily into the putative ligand binding site [1] of the V γ 9/V δ 2 T cell receptor, and a number of hydrogen bonds can be predicted enabling tight binding of the activator (unpublished observations). As V γ 9/V δ 2 T cells contribute to the immune response against some of the most harmful pathogens infecting humans, this information is of considerable relevance for immunotherapeutic approaches in diseases like tuberculosis and malaria.

Acknowledgements: This study was supported in part by the Bundesministerium für Bildung und Forschung (BioChance 0312588). Blood samples from healthy donors were kindly provided by the Institut für Klinische Immunologie und Transfusionsmedizin, Universität Gießen. We gratefully acknowledge the help and discussion of Erhard Dreiseidler, Rosel Engel, Christian Haug, Silke Sanderbrand, Irina Steinbrecher, Stefanie Wagner, Claus Weidemeyer, and Oliver Wolf at Jomaa Pharmaka GmbH, Giessen; Peter Reisenauer, Birgit Schleenbäcker-Lohfink, and Gertrud Stammeler at the Institut für

Organische Chemie, Universität Giessen; Gert Häde at the NMR Abteilung, Universität Marburg; Markus Böhm, Gerhard Klebe, and Martin Schlitzer at the Institut für Pharmazeutische Chemie, Universität Marburg; and the staff at BIM GmbH (Biotechnologie Gesellschaft Mittelhessen), Giessen.

References

- [1] Allison, T.J., Winter, C.C., Fournié, J.J., Bonneville, M. and Garboczi, D.N. (2001) *Nature* 411, 820–824.
- [2] Constant, P., Davodeau, F., Peyrat, M.A., Poquet, Y., Puzo, G., Bonneville, M. and Fournié, J.J. (1994) *Science* 264, 267–270.
- [3] Tanaka, Y., Morita, C.T., Tanaka, Y., Nieves, E., Brenner, M.B. and Bloom, B.R. (1995) *Nature* 375, 155–158.
- [4] Espinosa, E., Belmont, C., Sicard, H., Poupot, R., Bonneville, M. and Fournié, J.J. (2001) *Microbes Infect.* 3, 645–654.
- [5] Morita, C.T., Lee, H.K., Wang, H., Li, H., Mariuzza, R.A. and Tanaka, Y. (2001) *J. Immunol.* 167, 36–41.
- [6] Jomaa, H., Feurle, J., Luhs, K., Kunzmann, V., Tony, H.P., Herderich, M. and Wilhelm, M. (1999) *FEMS Immunol. Med. Microbiol.* 25, 371–378.
- [7] Altincicek, B., Moll, J., Campos, N., Foerster, G., Beck, E., Hoeffler, J.F., Grosdemange-Billiard, C., Rodríguez-Concepción, M., Rohmer, M., Boronat, A., Eberl, M. and Jomaa, H. (2001) *J. Immunol.* 166, 3655–3658.
- [8] Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H.K., Soldati, D. and Beck, E. (1999) *Science* 285, 1573–1576.
- [9] Rohmer, M. (1999) *Nat. Prod. Rep.* 16, 565–574.
- [10] Boucher, Y. and Doolittle, W.F. (2000) *Mol. Microbiol.* 37, 703–716.
- [11] Kabelitz, D., Glatzel, A. and Wesch, D. (2000) *Int. Arch. Allergy Immunol.* 122, 1–7.
- [12] Sicard, H. and Fournié, J.J. (2000) *Infect. Immun.* 68, 4375–4377.
- [13] Lange, B.M., Rujan, T., Martin, W. and Croteau, R. (2000) *Proc. Natl. Acad. Sci. USA* 97, 13172–13177.
- [14] Eisenreich, W., Rohdich, F. and Bacher, A. (2001) *Trends Plant Sci.* 6, 78–84.
- [15] Altincicek, B., Kollas, A.K., Sanderbrand, S., Wiesner, J., Hintz, M., Beck, E. and Jomaa, H. (2001) *J. Bacteriol.* 183, 2411–2416.
- [16] Altincicek, B., Kollas, A.K., Eberl, M., Wiesner, J., Sanderbrand, S., Hintz, M., Beck, E. and Jomaa, H. (2001) *FEBS Lett.* 499, 37–40.
- [17] Campos, N., Rodríguez-Concepción, M., Seemann, M., Rohmer, M. and Boronat, A. (2001) *FEBS Lett.* 488, 170–173.
- [18] Cunningham Jr., F.X., Lafond, T.P. and Gantt, E. (2000) *J. Bacteriol.* 182, 5841–5848.
- [19] Vaeth, E., Sladek, P. and Kenar, K. (1987) *Fresenius J. Anal. Chem.* 329, 584–589.
- [20] Piotto, M., Saudek, V. and Slenár, V. (1992) *J. Biomol. NMR* 2, 661–665.
- [21] Slenár, V., Piotto, M., Leppik, R. and Saudek, V. (1993) *J. Magn. Reson. (A)* 102, 241–245.
- [22] Greene, A.E., Coelho, F., Depres, J.P. and Brocksom, T.J. (1985) *J. Org. Chem.* 50, 1973–1975.
- [23] Koppisch, A.T., Blagg, B.S.J. and Poulter, C.D. (2000) *Org. Lett.* 2, 215–218.
- [24] Turek, T.C., Gaon, I. and Distefano, M.D. (2001) *J. Org. Chem.* 66, 3253–3264.
- [25] States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982) *J. Magn. Reson.* 48, 286–292.
- [26] Jackson, W.R., Moffat, M.R., Perlmutter, P. and Tasdelen, E.E. (1992) *Aust. J. Chem.* 45, 823–834.
- [27] Turek, T.C., Gaon, I. and Distefano, M.D. (1996) *Tetrahedron Lett.* 37, 4845–4848.
- [28] Itoh, T., Mitsukura, K., Kaihatsu, K., Hamada, H., Takagi, Y. and Tsukube, H. (1997) *J. Chem. Soc. Perkin Trans. 1*, 2275–2278.
- [29] Duvold, T., Cali, P., Bravo, J.M. and Rohmer, M. (1997) *Tetrahedron Lett.* 38, 6181–6184.
- [30] Poquet, Y., Constant, P., Halary, F., Peyrat, M.A., Gilleron, M.,

- Davodeau, F., Bonneville, M. and Fournié, J.J. (1996) *Eur. J. Immunol.* 26, 2344–2349.
- [31] Belmant, C., Espinosa, E., Poupot, R., Peyrat, M.A., Guiraud, M., Poquet, Y., Bonneville, M. and Fournié, J.J. (1999) *J. Biol. Chem.* 274, 32079–32084.
- [32] Chattopadhyay, S.K. and Pattenden, G. (2000) *J. Chem. Soc. Perkin Trans. 1*, 2429–2454.
- [33] Feurle, J., Espinosa, E., Eckstein, S., Pont, F., Kunzmann, V., Fournié, J.J., Herderich, M. and Wilhelm, M. (2001) *J. Biol. Chem.* (in press).
- [34] Pont, F., Luciani, B., Belmant, C. and Fournié, J.J. (2001) *Anal. Chem.* 73, 3562–3569.
- [35] Charon, L., Hoeffler, J.F., Pale-Grosdemange, C., Lois, L.M., Campos, N., Boronat, A. and Rohmer, M. (2000) *Biochem. J.* 346, 737–742.