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Irreversible inhibition of IspG, a target for the development of new antimicrobials, by a 2-vinyl analogue of its MEcPP substrate

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This paper is dedicated to the memory of our dear colleague and friend Dr. Jean-Luc Ferrer who sadly passed away in April 2020.

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Abstract: IspG (also called GcpE) is an oxygen-sensitive [4Fe-4S] enzyme catalyzing the penultimate step of the methylerythritol phosphate (MEP) pathway, a validated target for drug development. It converts 2-C-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP) into (*E*)-4-hydroxy-3-methyl-but-2-enyl-1-diphosphate (HMBPP). The reaction, assimilated to a reductive dehydration, involves redox partners responsible for the formal transfer of two electrons to substrate MEcPP. The 2-vinyl analogue of MEcPP was designed to generate conjugated species during enzyme catalysis, with the aim of providing new reactive centers to be covalently trapped by neighbouring amino acid residues. The synthesized substrate analogue displayed irreversible inhibition towards IspG. Furthermore, we showed that electron transfer occurs prior to inhibition, which might designate conjugated intermediates as probable affinity tags through covalent interaction at the catalytic site. This is the first report of an irreversible inhibitor of the IspG metalloenzyme.

Introduction

Isoprenoids form a large and structurally diversified class of natural products, which are biosynthesized from only two possible building blocks IPP **1** and DMAPP **2** (Figure 1).^[1] The so-called mevalonate (MVA) pathway was accepted earlier as the unique biosynthetic plan to provide these universal precursors of isoprenoids in all living organisms.^[2] Nevertheless, an alternative route was discovered in the early 1990's, which uses methylerythritol phosphate (MEP) as a key intermediate (Figure 1A).^[3] Interestingly, whereas the mevalonate pathway occurs mostly in eukaryotes, the MEP pathway is used exclusively by important pathogens and is absent in humans. A cascade of seven enzymatically catalyzed reactions occurs along the MEP pathway to generate **1** and **2** starting from pyruvate and glyceraldehyde-3-phosphate (Figure 1A).^[4]

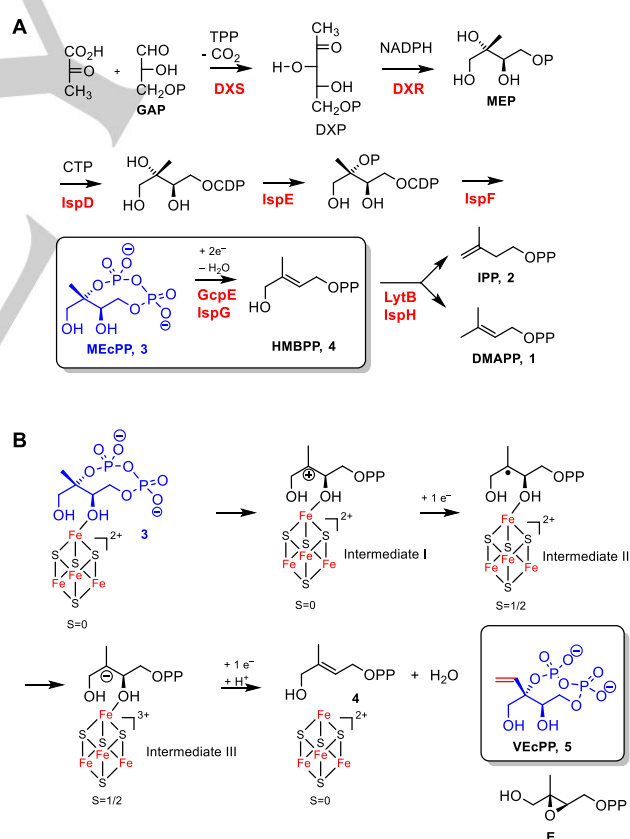


Figure 1. Methylerythritol phosphate pathway (A) and proposed mechanism for IspG (B).

Over the past three decades, an intense research activity was devoted not only to the discovery of all enzymes of the MEP pathway but also to the elucidation of the enzyme mechanisms and the resolution of the structures with the ultimate aim of generating potent inhibitors for the development of unprecedented and safe therapeutics to fight against

multiresistant bacteria and the malaria parasite *Plasmodium falciparum*.^[4,5] The five first steps of the MEP pathway, those going from pyruvate to 2-*C*-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP, **3**) are rather well documented.^[4] The two last stages converting MEcPP into IPP and DMAPP represent, however, a bottleneck in the full elucidation of the whole pathway and are respectively catalyzed by IspG and IspH (Figure 1A). Both proteins contain an oxygen sensitive [4Fe-4S] center and are therefore very difficult to handle, making these enzymes almost unexplored targets. IspG (also named GcpE) is the sixth enzyme of the MEP pathway, catalyzing the conversion of 2-*C*-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP) **3** into (*E*)-4-hydroxy-3-methyl-but-2-enyl-1-diphosphate (HMBPP) **4** via reductive deoxygenation (Figure 1).^[6] Although some aspects of the transformation have been firmly established, (such as the presence in the catalytic site of a [4Fe-4S] cofactor) the exact mechanism of IspG still remains elusive. However, the formal leaving of the cyclopyrophosphate ester at C-2 suggests possible formation of a carbocationic species as an initial intermediate. The carbocation could evolve towards the corresponding radical and carbanion via sequential electron transfers. A transient epoxide (structure **E**, Figure 1) has also been proposed as primary intermediate, resulting from nucleophilic displacement of the leaving phosphate.^[6d,6f] Characterization of these possible intermediates (I-III), mostly as bioinorganic species linked to the [4Fe-4S] cluster (Figure 1B), has been attempted recently using enzymology and isotopic-exchange experiments, EPR spectroscopies and X-ray crystallography.^[7] Intensive EPR/ENDOR investigations highlighted the formation of the paramagnetic anionic intermediate III or of the corresponding ferroxetane^[7d,7e,] but, most probably due to its transient nature, the radical intermediate II has never been observed. Exploitation of mechanistic proposals to design mechanism-based inhibitors of IspG has not been envisioned yet. Herein, we designed the vinyl-analogue of MEcPP (compound **5**, Figure 1) as a possible substrate, which could enter the first steps of the transformation to form the corresponding conjugated intermediates (carbocation, radical or carbanion). Delocalization would shift reactivity away from C-2 and provide new reactive centers which might be covalently trapped by neighbouring amino acid residues. The synthesis of 2-*C*-vinyl-D-erythritol cyclodiphosphate **5** is presented here as well as its evaluation as a substrate/inhibitor for IspG.

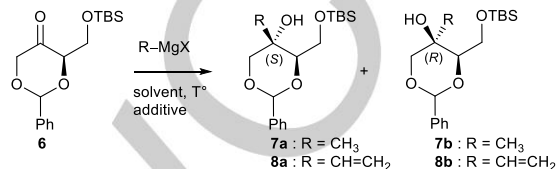
Results and Discussion

Synthesis of 2-*C*-vinyl-D-erythritol-2,4-cyclodiphosphate **5**

Targeted 2-vinylethritol cyclodiphosphate **5** was prepared via the dioxanone approach developed by Coates for the synthesis of MEcPP.^[8] A diastereoselective addition of vinylmagnesium bromide to dioxanone **6** (prepared from D-galactose in 5 steps, see Supporting Information), would fix the key tertiary alcohol. However, whereas the addition of MeMgBr proved almost completely selective towards the expected D-erythritol **7a** (Table 1, entry i), poor diastereoselection occurred with the vinyl nucleophilic species (Table 1, entry ii). Attempts to increase the

ratio in favour of **8a** by varying solvent or temperature failed.^[9] The presence of ZnCl₂ favored the unwanted isomer **8b**. Nevertheless, both isomers **8a/8b** could be easily separated by chromatography (47% overall yield) and we did not investigate further improvements at this stage. However, an unambiguous determination of the configuration at C-2 was essential before entering the next steps in order to secure the D-erythro series of all intermediates and that of final **5**.

Table 1. Diastereoselectivity of the addition to **6**.



| entry | -R | solvent | T° | dr (S/R) [a] |
|-------|---------------------|---------------------------------|--------|--------------|
| i | -CH ₃ | Et ₂ O | -78 °C | 95 : 5 [b] |
| ii | -CH=CH ₂ | Et ₂ O | -78 °C | 45 : 55 [c] |
| iii | -CH=CH ₂ | Et ₂ O | 0 °C | 43 : 57 |
| iv | -CH=CH ₂ | PhMe | -78 °C | 43 : 57 |
| v | -CH=CH ₂ | CH ₂ Cl ₂ | -78 °C | 47 : 53 |
| vi[d] | -CH=CH ₂ | CH ₂ Cl ₂ | -78 °C | 25 : 75 |
| vii | -CH=CH ₂ | CH ₂ Cl ₂ | 0 °C | 50 : 50 |

[a] As deduced from ¹H NMR of the crude reaction mixture. [b] 73% isolated yield. [c] 47% isolated yield. [d] In the presence of ZnCl₂.

To this aim, chemical correlation with the known ethylethritol triacetate **9** was achieved (Figure 2 and Supporting Information).^[10] After TBS-deprotection (TBAF), hydrogenation (H₂, Pd-C) and acetylation (Ac₂O), the triacetate resulting from **8a** had spectroscopical data (NMR and optical rotation) consistent with those described in the literature for **9**. The threitol epimer **10** afforded dissimilar signal patterns in NMR. The relative configuration at C-2 was also confirmed by NOESY experiments.

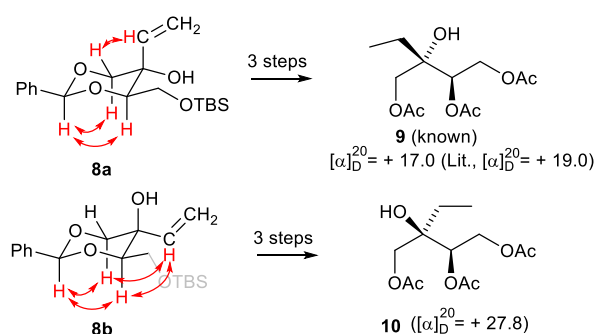
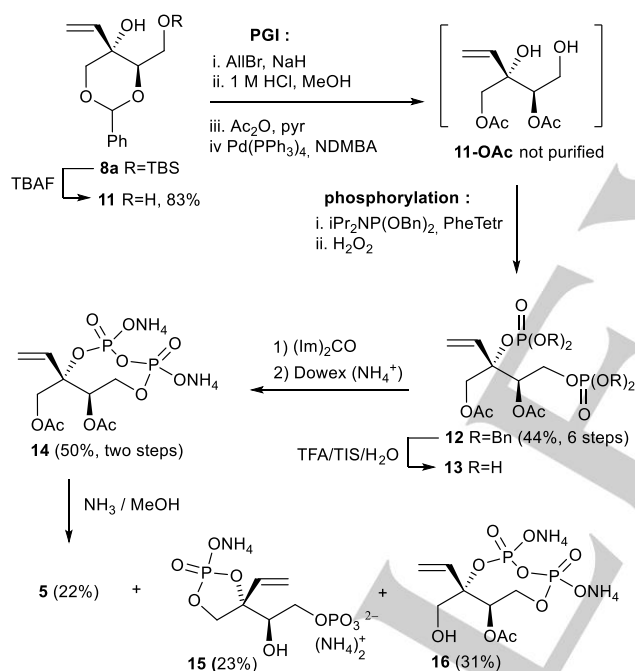


Figure 2. Configuration determination of **8a,b** via chemical correlation and NOEs.

Due to sensitivity of both the vinyl and the cyclic pyrophosphate to classical benzylidene deprotection procedures (hydrogenolysis or acidic cleavage) a Protecting Group Interconversion (PGI) towards acetates was accomplished in the next steps of the synthesis (Scheme 1). Diol **11** was tentatively allylated, followed by hydrolysis of the benzylidene acetal and acetylation under classical conditions. Then, de-allylation was performed smoothly by subsequent treatment with Pd(PPh₃)₄ at 40 °C for 2 h, in the presence of *N,N*-dimethylbarbituric acid

(NDMBA) as an allyl scavenger,^[11] to afford intermediate **11-OAc**. Migration issues of the secondary acetate to the neighbouring primary –OH during purification forced us to use **11-OAc** as crude material in the next step. Bis-phosphorylation of diol **11-OAc** was performed *via* the phosphoramidite method using a slight excess (3 equiv) of dibenzyl *N,N*-diisopropylphosphoramidite and hydrogen peroxide as the oxidant. *m*CPBA traditionally used in this latter step gave improper results due to additional reaction with the vinyl functionality. Orthogonal deprotection of the P(O)Bn esters, which preserved integrity of the double bond, was achieved with trifluoroacetic acid/triisopropylsilane/water 96:2:2.^[12] Bis-phosphoric acid **13** was then treated with carbonyl-diimidazole as the coupling agent to obtain the corresponding cyclic pyrophosphate **14** after ion exchange chromatography. Final saponification of acetates proved difficult and several conditions were tested to improve the yields. Such protecting groups have been used in a synthesis of MEcPP and were removed with success by using aqueous ammonia.^[13a] However, under these same conditions rearrangement product **15** was obtained as the only isolable material from vinyl analogue **14**.



Scheme 1. Synthesis of VEcPP **5**.

Such a base-promoted transformation of the cyclodiphosphate moiety has already been observed with MEcPP.^[13] Nevertheless, treatment with diluted methanolic ammonia alleviated this unwanted process and a fine control of the reaction time afforded acceptable amount of the targeted vinylcyclodiphosphate **5** along with **15** and a partially acetylated intermediate **16**. Chromatographic separation afforded pure VEcPP **5**, which was used as its ammonium salt in the biological assays. Structure of **5** was assessed by HRMS and NMR data. In particular, integrity of the cyclodiphosphate ester was confirmed by the 23.6 Hz ²J_{P-P} coupling constant.

Enzymatic assays

E. coli IspG harbours an oxygen-sensitive [4Fe-4S]²⁺ cluster in its catalytic site, that is held by three conserved cysteines and an additional non-cysteine ligand. The nature of this ligand was not unequivocally assessed, but Mössbauer spectroscopy data and crystal structures of *Aquifex aeolicus* and *Thermus thermophilus* homologues suggest the presence of a glutamate residue.^[14]

Due to its oxygen sensitivity, *E. coli* IspG needs to be handled in a glove box under strictly inert (N₂) atmosphere. Production and purification of IspG was effected following our previously described procedure,^[6b] with some improvements (see Experimental section). UV/visible spectrum as well as iron and sulfur content of the protein thus obtained were similar to those previously reported,^[6b,6e,15a] ascertaining the presence of the obligate [4Fe-4S]²⁺ cluster. Only few analytical solutions exist to monitor the IspG-catalyzed reaction. IspG, as an interconnected enzymatic complex, promotes the conversion of **3** into **4** in the presence of an external reduction system. In *E. coli*, the natural flavodoxin (FidA)/flavodoxin reductase (FpR1)/NADPH system plays this role (Figure 3).^[15b] Hence, enzyme activity was evaluated by monitoring NADPH consumption at 340 nm in the presence of optimized concentrations of this reducing system. Under these conditions, activity of IspG was 45 nmol/min/mg (Figure 3, blue), in good agreement with previous data.^[15] Progress curves showed typical profiles over a 15-minute observation period characterized by sharp drop in NADPH concentration attributable to IspG turn-over, followed by a low-sloping linear region at t > 15 min due to sole spontaneous NADPH degradation at pH 8.

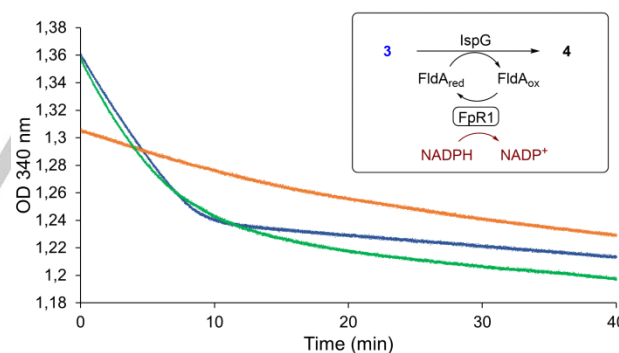


Figure 3. Enzymatic assay : decrease of the absorbance of NADPH at 340 nm in the presence and absence of VEcPP **5**. Conditions: NADPH (2 mM), FidA (30 μM), FpR1 (17 μM), IspG (12 μM) and VEcPP (0 μM, dark blue or 500 μM red) in 50 mM Tris HCl buffer pH 8 at 37°C, initiating the reaction by addition of MEcPP (250 μM) after 10 min pre-incubation at 37 °C; no VEcPP-IspG initial contact period: the reaction is initiated by addition of IspG (green). Samples were prepared in the glove box, and additions were performed with a gastight syringe.

Preliminary experiments were carried out to test the ability of VEcPP **5** to act either as substrate or inhibitor. Incubation of 500 μM of **5** in the presence of natural substrate **3** (250 μM), IspG and reduction system led to a marked decrease in the activity of IspG, revealing the inhibitor character of the vinyl analogue (Figure 3, red). Interestingly, dissimilar progress curves were obtained depending whether substrate **3** or IspG was used to initiate the IspG reaction, while the linear regions at t > 25 min of both progress curves display the same slope indicating same steady-state rates (Figure 3, red and green curves). This

behaviour showing that the initial velocity decreased only after extended initial enzyme-inhibitor contact period (Figure 3, red) suggests slow binding kinetics.^[16]

Further experiments were conducted to determine IC_{50} value of VECP 5, at a fixed substrate 3 concentration of 250 μ M. Due to the slow-binding behaviour, preincubation of IspG with inhibitor 5 in the presence of the reduction system was carried out for 30 min, prior to addition of substrate 3 to initiate the reaction. Kinetic data afforded an apparent IC_{50} of 62.7 \pm 2.1 μ M (Figure 4). Reducing the initial contact period between IspG and VECP 5 to 10 min had a clear impact on inhibition potency (IC_{50} = 96.2 μ M). The observed increase of the potency of inhibitor 5 in function of the pre-incubation time with IspG might indicate covalent inhibition, as irreversible interactions might slowly form between the inhibitor and IspG and as the rate of enzyme inactivation due to covalent bond formation is usually slow on the time scale of enzyme turnover. Furthermore, slow-binding kinetics might be found with either reversible or irreversible inhibitors as a consequence of slow establishment of enzyme-inhibitor equilibrium.

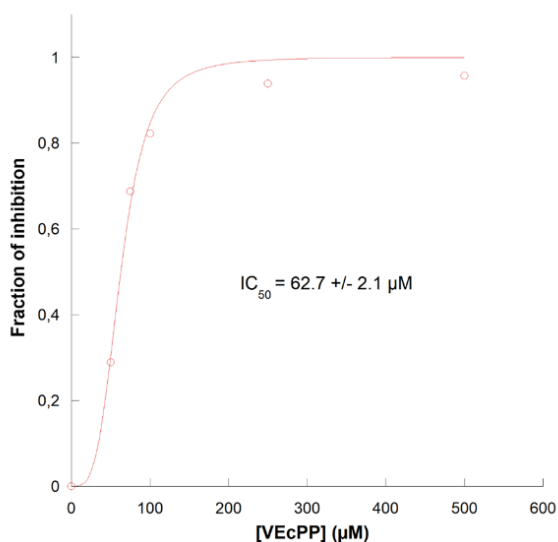


Figure 4. Fraction of inhibition of IspG versus VECP 5 concentrations. Conditions: NADPH (2 mM), FldA (30 μ M), FpR1 (17 μ M), MEcPP 3 (250 μ M) and IspG (12 μ M) in 50 mM Tris HCl buffer pH 8 and the following concentrations of VECP: 0 μ M, 50 μ M, 75 μ M, 100 μ M, 250 μ M and 500 μ M. All the samples were pre-incubated for 30 min at 37°C before MEcPP was added with a gastight syringe to initiate the reaction.

We thus explored the possibility for VECP 5 to act as an irreversible inhibitor (Figure 5).^[17] For this purpose, IspG was incubated with enough VECP 5 to achieve complete inactivation, in the presence of the reduction system (medium 1). Inactivation was confirmed by an enzymatic assay using an aliquot of this mixture (Figure 5, blue), which showed no NADPH consumption. Then, several centrifugation-dilution cycles of medium 1 using a centrifugal filter (Amicon Ultra 10K) were carried out to remove unbound 5, affording medium 2. Such a process is known to disassociate reversible inhibitors from their enzyme target.^[18] Here, the IspG was still inactive after removal of unbound 5 (Figure 5, green) whereas IspG, in the control performed under the same conditions but omitting VECP 5, retained its activity. This result afforded evidence for irreversible inactivation of IspG by VECP 5.

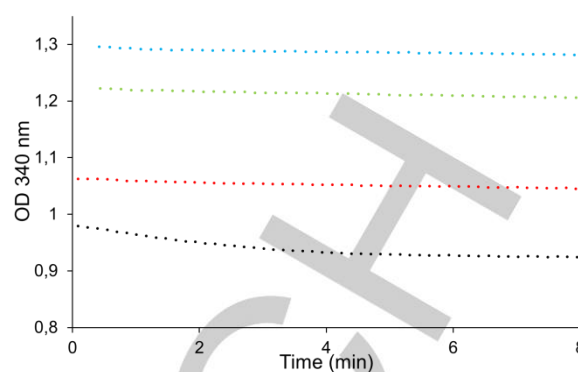


Figure 5. Assays to monitor the irreversibility of the inhibition. Decrease of the absorbance of NADPH at 340 nm before removing the unbound 5 (blue); after removing unbound 5 (green); after removing unbound 5 and adding some new amounts of FpR1 and FldA in the assay (red); after removing unbound 5 and adding active IspG in the assay (black).

Further control experiments were conducted with medium 2 to assess that 5 does specifically interfere with IspG and not with the reduction system. To this aim, additional amount of flavodoxin and flavodoxin reductase was added to the enzymatic assay used to test the activity of IspG present in medium 2, but no gain in enzyme velocity was observed (Figure 5, red). A control experiment in which IspG was omitted from the preincubation further confirmed that the reduction system remained fully active in presence of VECP 5 (Figure S3). However, IspG activity could only be recovered using medium 2 by adding an aliquot of active IspG in the assay (Figure 5, black), which designated IspG as the protein target of VECP 5.

To further confirm the irreversible mode of inhibition, high substrate concentrations were added to inactivated IspG in order to check if the substrate can compete out the inhibitor as in case of competitive inhibition. Therefore, IspG was incubated in presence of the reduction system with a fixed VECP 5 concentration of 110 μ M to achieve approximately 75% of inhibition. After removal of unbound VECP 5, the activity of IspG in the remaining solution stayed stable by varying the substrate concentration from 250 μ M to 1 mM confirming the irreversible mode of inhibition (Figure S4). The decrease in activity seen after 10 min of reaction in the IspG assay using 250 μ M of substrate is due to substrate depletion (Figure S4, orange).

Interestingly, a sample of IspG preincubated with 5 in the absence of the reduction system retained its activity after removing the inhibitor from the reaction mixture. This experiment shows that the flavodoxin/flavodoxin reductase/NADPH system is required during preincubation to achieve IspG inhibition, suggesting that VECP 5 might enter the first steps of the enzymatic reaction to promote inhibition.

This finding was confirmed by replacing the natural substrate MEcPP 3 by VECP 5 in the IspG assay (Figure 6).

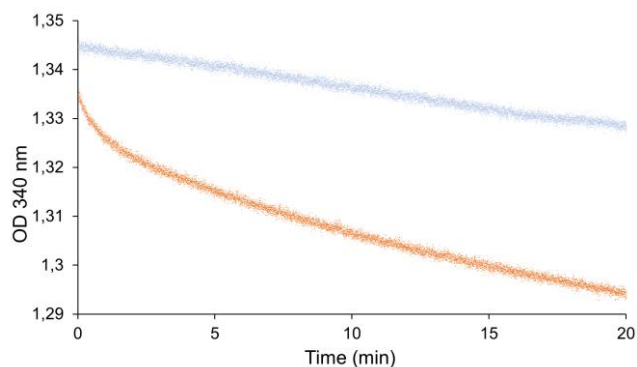


Figure 6. Decrease of the absorbance of NADPH at 340 nm in the IspG assay testing **5** as substrate of IspG. Conditions: NADPH (2 mM), FldA (30 μ M), FpR1 (17 μ M) and 500 μ M VEcPP in 50 mM Tris-HCl buffer pH 8 in presence of IspG (12 μ M) (orange) and absence of IspG (blue).

We observed that IspG was active in the first minutes of reaction in the presence of VEcPP as the only possible substrate, then the activity slowly decreased and after 15 minutes of reaction the enzyme was almost totally inhibited. These data indicate that VEcPP enters the active site and may undergo electron transfers before inhibiting the enzyme.

Conclusion

In conclusion, we have reported the synthesis of the first substrate analogue of MEcPP, a key intermediate from the MEP pathway. Inhibitor **5**, which features a cyclodiphosphate moiety, was prepared from D-galactose via multistep synthesis using Protective Group Interconversion methodology to allow orthogonal deprotection of both phosphate and alcohol protecting groups in the presence of the obligate vinyl functionality. Biological results highlight that VEcPP acts as an irreversible inhibitor of *E. coli* IspG and that electron capture might be needed to chemically transform VEcPP **5** into the reactive species responsible for enzyme inactivation. Further investigations are currently in progress to determine the exact mechanism of action of VEcPP through the identification of molecular modifications of the initial E-I complex.

Experimental Section

General methods

Reactants and reagents were purchased from standard suppliers (Sigma-Aldrich, Alfa-Aesar, Fisher Scientific) and were used without further purification. Methanol, dichloromethane, diethyl ether, toluene and tetrahydrofuran were dried on an Innovative Technology, Inc. Pure Solv MD system; other solvents were dried according to conventional methods. Reactions were monitored by TLC using Silica gel F254 (0.2 mm) TLC plates, detection being carried out by spraying with an aqueous solution of KMnO_4 (2%) / Na_2CO_3 (4%) or alcoholic solutions of phosphomolybdic acid or p-anisaldehyde, followed by heating. Column chromatography purifications were performed over silica gel M 9385 (40–63 μ m) Kieselgel 60. NMR spectra were recorded on Bruker AC 250 (250 MHz for ^1H , 63 MHz for ^{13}C and 101 MHz for ^{31}P), 500 (500 MHz for ^1H , 126 MHz for ^{13}C and 202 MHz for ^{31}P) or 600 (600 MHz for ^1H , 150 MHz for ^{13}C and 202

MHz for ^{31}P) spectrometers. Chemical shifts are expressed in parts per million (ppm) and were calibrated to deuterated or residual non-deuterated solvent peaks for ^1H and ^{13}C spectra. Coupling constants are in Hz and splitting pattern abbreviations are: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet. DEPT and JMOD 1D NMR experiments, COSY, HSQC, and HMBC 2D NMR experiments were used to confirm the NMR peak assignments for all compounds. Optical rotations were determined at 20 $^\circ\text{C}$ (unless otherwise stated) with a Perkin-Elmer Model 241 polarimeter. High Resolution Mass Spectra (HRMS) were performed on Q-TOF Micro micromass positive ESI (CV = 30 V).

1,3-O-Benzylidene-2-C-vinyl-D-erythritol 4(*t*-Butyldimethylsilyl) Ether, **8a**

Under argon, a solution of 1,3-O-benzylidene-D-erythrose 4(*t*-Butyldimethylsilyl) ether **6** (1.54 mmol, 1 equiv) in dry diethyl ether (32 mL) was stirred and cooled at -78°C as vinylmagnesium bromide 1M in diethyl ether (9.2 mmol, 9.2 mL, 6 equiv) was added dropwise (TLC monitoring with $\text{Et}_2\text{O}/\text{PE}$, 1:9). After 1h30, methanol and saturated NH_4Cl were added and the solution was warmed up to room temperature. The product was extracted with ethyl acetate, the extracts were dried over MgSO_4 and finally evaporated. Purification by flash chromatography (SiO_2 , petroleum ether/diethyl ether 95:5) afforded **8b** as the less polar isomer (132 mg, 0.38 mmol, 24%) and the targeted more polar **8a** (123 mg, 0.35 mmol, 23% over 2 steps) as yellows oils.

8a, ^1H NMR (CDCl_3 , 500 MHz): δ 0.08 (s, 3H, CH_3), 0.09 (s, 9H, *t*-Bu), 3.74–3.82 (m, 2H, H_a), 3.84 (d, 1H, $J_{\text{H-H}} = 10.6\text{Hz}$, H_{1a}), 3.95 (dd, 1H, $J_{\text{H-H}} = 5\text{Hz}$ and 9.9Hz , H_3), 3.99 (d, 1H, $J_{\text{H-H}} = 10.6\text{Hz}$, H_{1b}), 5.38 (dd, 1H, $J_{\text{H-H}} = 1.9\text{Hz}$ and 10.9Hz , $\text{CH}=\text{CH}_a\text{H}_b$), 5.56 (dd, 1H, $J_{\text{H-H}} = 1.9\text{Hz}$ and 17.4Hz , $\text{CH}=\text{CH}_a\text{H}_b$), 5.60 (s, 1H, CHPh), 6.53 (dd, 1H, $J_{\text{H-H}} = 10.9\text{Hz}$ and 17.4Hz , $\text{CH}=\text{CH}_2$), 7.35–7.40 (m, 3H, Ph), 7.50–7.56 (m, 2H, Ph). ^{13}C NMR (CDCl_3 , 126 MHz): δ -5.6 (CH_3), -5.6 (CH_3), 18.2 (Cq, *t*-Bu), 25.9 (*t*-Bu), 63.8 (C_4), 69.6 (Cq, C_2), 76.0 (C_1), 80.3 (C_3), 102.1 (CHPh), 115.0 ($\text{CH}=\text{CH}_2$), 126.3 (Ph), 128.4 (Ph), 129.2 (Ph), 137.6 (Cq, Ph), 137.7 ($\text{CH}=\text{CH}_2$). HRMS (ESI $^+$) Calcd for $\text{C}_{19}\text{H}_{30}\text{O}_4\text{NaSi}$ (M + Na) $^+$, 373.1811, Found: 373.1803. $[\alpha]_D^{25} = -13.1^\circ$ (c 0.7, CHCl_3).

1,3-O-Benzylidene-2-C-vinyl-D-erythritol, **11**

An anhydrous solution of 1,3-O-benzylidene-2-C-vinyl-D-erythritol, 4(*t*-Butyldimethylsilyl) ether **8a** (116 mg, 0.33 mmol, 1 equiv) in 0.8 mL tetrahydrofuran was stirred at 0°C and tetrabutylammonium fluoride 1M in tetrahydrofuran (0.40 mL, 0.40 mmol, 1.2 equiv) was added dropwise. The solution was brought to room temperature and stirring for 30 min. Water was added and the mixture was extracted with ethyl acetate, the extracts were dried over MgSO_4 and evaporated to give the crude product as a yellow oil. Purification by flash chromatography (SiO_2 , petroleum ether/ethyl acetate 7:3) gave 1,3-O-benzylidene-2-C-vinyl-D-erythritol **11** as a colourless powder (65 mg, 0.28 mmol, 83%).

^1H NMR (CDCl_3 , 500 MHz): δ 3.77 (dd, 1H, $J_{\text{H-H}} = 6.6\text{Hz}$ and 11Hz , H_{4a}), 3.82–3.84 (m, 2H, $\text{H}_{1a} + \text{H}_{4b}$), 3.81–3.84 (m, 2H, $\text{H}_{1b} + \text{H}_3$), 5.37 (dd, 1H, $J_{\text{H-H}} = 1.1\text{Hz}$ and 11Hz , $\text{CH}=\text{CH}_a\text{H}_b$), 5.49 (dd, 1H, $J_{\text{H-H}} = 1.2\text{Hz}$ and 17.5Hz , $\text{CH}=\text{CH}_a\text{H}_b$), 5.61 (s, 1H, CHPh), 6.53 (dd, 1H, $J_{\text{H-H}} = 11\text{Hz}$ and 17.5Hz , $\text{CH}=\text{CH}_2$), 7.36–7.41 (m, 3H, Ph), 7.49–7.51 (m, 2H, Ph). ^{13}C NMR (CDCl_3 , 126 MHz): δ 62.0 (C_4), 68.6 (Cq, C_2), 76.2 (C_1), 82.7 (C_3), 102.1 (CHPh), 114.7 ($\text{CH}=\text{CH}_2$), 126.4 (Ph), 128.5 (Ph); 129.4 (Ph), 137.3 ($\text{CH}=\text{CH}_2$), 137.4 (Cq, Ph). HRMS (ESI $^+$) Calcd for $\text{C}_{13}\text{H}_{16}\text{O}_4\text{Na}$ (M + Na) $^+$, 259.0946, Found: 259.0949. $[\alpha]_D^{25} = -3.6^\circ$ (c 0.7, CHCl_3).

Protective group interconversion/phosphorylation procedures from **11** to **12**

2,4-di-O-Allyl-1,3-O-benzylidene-2-C-vinyl-D-erythritol

An anhydrous solution of 1,3-O-benzylidene-2-C-vinyl-D-erythritol **11** (92 mg, 0.39 mmol, 1 equiv) in 1.1 mL of *N,N*-dimethylformamide was stirred at 0°C as NaH 60% (62 mg, 1.55 mmol, 3.8 equiv) was added by portions followed by allyl bromide dropwise (0.13 mL, 1.49 mmol, 3.8 equiv). The solution was stirred for 3h at room temperature before adding methanol and water. The product was extracted with ethyl acetate, the extracts were dried over MgSO_4 and evaporated to give the crude product as an oil. Purification by flash chromatography (SiO_2 , petroleum

ether/ethyl acetate 9:1) gave the desired product in 89% yield (110 mg, 0.35 mmol) as a colourless oil.

¹H NMR (CDCl₃, 250 MHz) : δ 3.48 (dd, 1H, J_{H-H} = 8.2Hz and 10.9Hz, H_{4a}), 3.88-3.94 (m, 3H, H_{1a}+H_{4b}+C₂-O-CH_aH_b), 4.03-4.09 (m, 4H, H₃+ C₄-O-CH₂+ C₂-O-CH_aH_b), 4.35 (d, 1H, J_{H-H} = 10.6Hz, H_{1b}), 5.19 (d, 2H, J_{H-H} = 10.5Hz, 2 x CH_aH_b allyl), 5.28 (dd, 1H, J_{H-H} = 1.8Hz and 7.1Hz, CH_aH_b allyl), 5.35 (dd, 1H, J_{H-H} = 1.8Hz and 7.1Hz, CH_aH_b allyl), 5.45 (dd, 2H, J_{H-H} = 1.4Hz and 11.2Hz, CH_aH_b vinyl), 5.55 (dd, 2H, J_{H-H} = 1.4Hz and 17.8Hz, CH_aH_b vinyl), 5.62 (s, 1H, CHPh), 5.83-6.01 (m, 2H, 2 x CH allyl), 6.09 (dd, 1H, J_{H-H} = 11.2Hz and 17.8Hz, CH vinyl), 7.36-7.40 (m, 3H, Ph), 7.51-7.55 (m, 2H, Ph). ¹³C NMR (CDCl₃, 63 MHz) : δ 63.5 (C₂-O-CH₂), 69.1 (C₄), 71.7 (Cq, C₂), 72.4 (C₄-O-CH₂), 73.1 (C₁), 83.2 (C₃), 101.7 (CHPh), 115.8 (C₂-O-CH₂-CH=CH₂), 117.0 (C₄-O-CH₂-CH=CH₂), 118.1 (CH₂ vinyl), 126.4 (Ph); 128.2 (Ph); 129.0 (Ph), 134.3 (CH vinyl), 134.8 (CH allyl); 135.0 (CH allyl), 137.7 (Cq, Ph). HRMS (ESI⁺) Calcd for C₁₉H₂₄O₄Na (M + Na)⁺, 339.1572, Found: 339.1570. [α]_D²⁵ = + 39.6° (c 0.4, CHCl₃).

2,4-di-O-Allyl-2-C-vinyl-D-erythritol

A solution of diallyloxy-benzylidene above (104 mg, 0.33 mmol, 1 equiv) in 9 mL of HCl 1N/MeOH 1:1 was stirred for 1h30 at 40°C and then evaporated. The crude diol was directly applied to the next step.

Alternatively, purification by flash chromatography (SiO₂, petroleum ether/ethyl acetate 65:35) afforded pure product as a colourless oil.

¹H NMR (CD₃OD, 500 MHz) : δ 3.39 (dd, 1H, J_{H-H} = 8.5Hz and 10.3Hz, H_{4a}), 3.74 (d, 1H, J_{H-H} = 12.1Hz, H_{1a}), 3.78 (dd, 1H, J_{H-H} = 2.2Hz and 10.3Hz, H_{4b}), 3.88 (d, 1H, J_{H-H} = 12.1Hz, H_{1b}), 3.99-4.05 (m, 5H, H₃+ C₂-O-CH₂+ C₄-O-CH₂), 5.10 (ddt, 1H, J_{H-H} = 1.5Hz and 2Hz and 10.5Hz, C₂-O-CH₂-CH=CH_aH_b), 5.15 (ddt, 1H, J_{H-H} = 1.3Hz and 1.9Hz and 10.5Hz, C₄-O-CH₂-CH=CH_aH_b), 5.23-5.31 (m, 4H, CH₂ vinyl+ C₂-O-CH₂-CH=CH_aH_b+ C₄-O-CH₂-CH=CH_aH_b), 5.73 (dd, 1H, J_{H-H} = 11.3Hz and 17.8Hz, CH vinyl), 5.87-5.98 (m, 2H, 2 x CH allyl). ¹³C NMR (CD₃OD, 126 MHz) : δ 62.4 (C₁), 64.8 (C₁-O-CH₂), 72.7 (C₄), 73.0 (C₄-O-CH₂), 73.1 (C₃), 82.3 (Cq, C₂), 115.5 (C₂-O-CH₂-CH=CH₂), 117.1 (C₄-O-CH₂-CH=CH₂), 117.9 (CH₂ vinyl), 136.2 (CH allyl), 136.5 (CH vinyl), 136.9 (CH allyl). HRMS (ESI⁺) Calcd for C₁₂H₂₀O₄Na (M + Na)⁺, 251.1259, Found: 251.1253.

1,3-di-O-Acetyl-2,4-di-O-allyl-2-C-vinyl-D-erythritol

The crude diol (0.33 mmol) in 2.4 mL of pyridine was stirred at room temperature and acetic anhydride (0.62 mL, 2.28 mmol) was added dropwise. The solution was stirred for 16 h before adding methanol and evaporating the resulting solution. Purification by flash chromatography (SiO₂, petroleum ether/ethyl acetate 9:1) gave the desired product as a colourless oil in 86% yield over two steps (88 mg, 0.28 mmol).

¹H NMR (CDCl₃, 250 MHz) : δ 2.04 (s, 3H, CH₃), 2.06 (s, 3H, CH₃), 3.50 (dd, 1H, J_{H-H} = 8.3Hz and 11Hz, H_{4a}), 3.75 (dd, 1H, J_{H-H} = 2.7Hz and 11Hz, H_{4b}), 3.88 (ddt, 1H, J_{H-H} = 1.4Hz and 5.5Hz and 13Hz, CH_aH_b-CH=CH₂), 3.96-4.04 (m, 3H, CH_aH_b-CH=CH₂+ 2 x CH_aH_b-CH=CH₂), 4.09 (d, 1H, J_{H-H} = 12.6Hz, H_{1a}), 4.30 (d, 1H, J_{H-H} = 12.6Hz, H_{1b}), 5.09-5.38 (m, 6H, CH₂ vinyl+2 x CH₂ allyl), 5.43 (dd, 1H, J_{H-H} = 2.7Hz and 8.3Hz, H₃), 5.71 (dd, 1H, J_{H-H} = 11.4Hz and 17.5Hz, CH vinyl), 5.75-5.92 (m, 2H, 2 x CH allyl). ¹³C NMR (CDCl₃, 63 MHz) : δ 21.0 (CH₃), 21.2 (CH₃), 62.7 (C₁), 64.4 (C₂-O-CH₂), 68.8 (C₄), 71.6 (C₃), 71.9 (C₄-O-CH₂), 79.0 (Cq, C₂), 115.7; 116.9; 118.7 (CH₂ vinyl+ 2 CH₂ allyl), 133.9 (CH vinyl), 134.7 (CH allyl), 134.8 (CH allyl), 170.0 (CO), 170.8 (CO). HRMS (ESI⁺) Calcd for C₁₆H₂₄O₆Na (M + Na)⁺, 335.1471, Found: 335.1459. [α]_D²⁵ = +30° (c 0.8, CHCl₃).

1,3-di-O-Acetyl-2,4-bis(dibenzylphosphate)-2-C-vinyl-D-erythritol 12

Under nitrogen atmosphere, 1,3-dimethylbarbituric acid (264 mg, 1.69 mmol, 6 equiv) and 2.1 mL of degassed dichloromethane were added to the diacetate above (72 mg, 0.23 mmol, 1 equiv). After addition of Pd(PPh₃)₄ (40 mg, 0.035 mmol, 0.15 equiv) the resulting solution was stirred for 2 h at 40°C to afford **11-OAc**, which was directly used in the next step. Dibenzyl *N,N*-diisopropylphosphoramidite (0.23 mL, 0.68 mmol, 3 equiv) followed by 5-phenyl-1H-tetrazole (101 mg, 0.69 mmol, 3 equiv) were added to the reaction mixture above and stirring was continued for 4 h. Finally, the solution was cooled to 0°C and H₂O₂ 30% (0.09 mL, 0.87 mmol, 3.8 equiv) was added and the resulting mixture was stirred at room temperature for 1h30. EtOAc was then added and the organic phase was subsequently washed twice with Na₂S₂O₃ 10%, once with saturated NaHCO₃ and once with brine. After drying over MgSO₄ and evaporation, the crude product was purified by flash chromatography (SiO₂, petroleum ether/ethyl acetate 5:5) to give the expected bisphosphate **12** as a yellow oil (102 mg, 0.14 mmol, 58%).

¹H NMR (CDCl₃, 500 MHz) : δ 1.92 (s, 3H, CH₃), 1.93 (s, 3H, CH₃), 4.05 (ddd, 1H, J_{H-H} = 7.9Hz and 11.4Hz, ³J_{P-H} = 7.8Hz, H_{4a}), 4.25 (ddd, 1H, J_{H-H} = 2.5 Hz and 11.4Hz, ³J_{P-H} = 6.9Hz, H_{4b}), 4.40 (d, 1H, J_{H-H} = 12.5Hz, H_{1a}), 4.56 (d, 1H, J_{H-H} = 12.5Hz, H_{1b}), 4.84-5.14 (m, 8H, 4 x CH₂-Ph), 5.35 (d, 1H, J_{H-H} = 11.3Hz, CH_aH_b vinyl), 5.42 (d, 1H, J_{H-H} = 17.5Hz, CH_aH_b vinyl), 5.58 (dd, 1H, J_{H-H} = 2.5 and 7.9Hz, H₃), 5.90 (ddd, 1H, J_{H-H} = 11.4 Hz and 17.5Hz, ³J_{P-H} = 1.5Hz, CH vinyl), 7.31-7.32 (m, 20H, Ph).

¹³C NMR (CDCl₃, 126 MHz) : δ 20.7 (CH₃), 20.8 (CH₃), 63.2 (C₁), 65.9 (d, ²J_{P-C} = 5.1Hz, C₄), 69.5-69.6 (m, 4 x CH₂-Ph), 71.3 (dd, ³J_{P-C} = 7.7Hz and 7.9Hz, C₃), 84.2 (d, ²J_{P-C} = 6.9Hz, Cq, C₂), 119.7 (CH₂ vinyl), 127.9 (Ph), 127.9 (Ph), 128.0 (Ph), 128.0 (Ph), 128.1 (Ph), 128.6 (Ph), 128.7 (Ph), 128.7 (Ph), 132.1 (d, ³J_{P-C} = 3.6 Hz, CH vinyl), 135.6 (d, ³J_{P-C} = 2.1Hz, Cq, Ph), 135.7 (d, ³J_{P-C} = 2.2Hz, Cq, Ph), 135.8 (d, ³J_{P-C} = 1.8Hz, Cq, Ph), 135.8 (d, ³J_{P-C} = 1.6Hz Cq, Ph), 169.5 (CO), 170.3 (CO). ³¹P NMR (CDCl₃, 101 MHz) : δ -6.1 (m, 1P), -1.0 (m, 1P). HRMS (ESI⁺) Calcd for C₃₈H₄₂O₁₂P₂Na (M + Na)⁺, 775.2049, Found: 775.2041.

1,3-di-O-Acetyl-2-C-vinyl-D-erythritol-2,4-cyclodiphosphate, diammonium salt 14

Benzoyloxyphosphate ester (125 mg, 0.17 mmol, 1 equiv) was dissolved in 2.3 mL of a solution of trifluoroacetic acid/triisopropylsilane/water 96:2:2 and the reaction was stirred for 4h30 and then concentrated. After several co-evaporation with toluene to remove all traces of trifluoroacetic acid the residue was washed twice with toluene and diethyl ether to give **13** (see NMR spectra of this intermediate **13** below) that was used as such in the next step. The solid (44 mg) was dissolved in 1.6 mL of DMSO and this mixture was added dropwise to a solution of 1,1'-carbonyldiimidazole (55 mg, 0.34 mmol, 3 equiv) in 1.1 mL of DMSO or CH₂Cl₂. After one night of stirring, the solvent was evaporated. The crude product was successively washed with Et₂O and CH₂Cl₂ and the residue was purified by ion exchange chromatography on a DOWEX 50WX8 (NH₄⁺) column, eluting with a solution of 0.2% NH₄HCO₃ and then purified by flash chromatography (SiO₂, CHCl₃/MeOH/NH₄OH 1M, 6:4:1) to afford **14** as an oil (23 mg, 0.056 mmol, 33% over two steps).

¹H NMR (D₂O, 600 MHz) : δ 2.13 (s, 3H, CH₃), 2.14 (s, 3H, CH₃), 4.08 (td, 1H, J_{H-H} = 2.5Hz and 12.9Hz, H_{4a}), 4.27 (ddd, 1H, J_{H-H} = 7.4 Hz and 12.9 Hz, ³J_{P-H} = 15.9Hz, H_{4b}), 4.45 (d, 1H, J_{H-H} = 11.6Hz, H_{1a}), 4.58 (d, 1H, J_{H-H} = 11.6Hz, H_{1b}), 5.49-5.53 (m, 2H, H₃+ CH_aH_b vinyl), 5.65 (d, 1H, J_{H-H} = 17.3Hz, CH_aH_b vinyl), 6.06 (ddd, 1H, J = 11.1 Hz and 17.3Hz, ⁴J_{P-H} = 3.9 Hz, CH vinyl). ¹³C NMR (D₂O, 151 MHz) : δ 20.2 (CH₃), 64.1 (d, ²J_{P-C} = 6.5 Hz, C₄), 66.1 (C₁), 71.5 (br s, C₃), 81.2 (d, ²J_{P-C} = 8 Hz, Cq, C₂), 119.2 (CH₂ vinyl), 132.9 (d, ³J_{P-C} = 9 Hz, CH vinyl), 172.4 (CO), 173.8 (CO). ³¹P NMR (D₂O, 202 MHz) : δ -16.7 (d, 1P, J = 23.9Hz), -11.6 (m, 1P). HRMS (ESI⁺) Calcd for C₁₀H₁₆O₁₁P₂Na (M + Na)⁺, 397.0066, Found: 397.0074. [α]_D²⁵ = -25.7° (c 0.5, MeOH).

2-C-Vinyl-D-erythritol-2,4-cyclodiphosphate, diammonium salt 5

The diacetate (111 mg, 0.27 mmol, 1 equiv) was dissolved in methanol (1 mL) and a solution of 7 M NH₃ in methanol (200 μL) was added. The resulting solution was stirred at 40 °C for 6 h and evaporated. The crude product was washed twice with CH₂Cl₂ to remove acetamide and the products were separated by flash chromatography (SiO₂, chloroform/methanol/ammonium acetate 1%, 5:5:1) to afford three fractions: a first crop of monoacetylated intermediate (31 mg, 0.085 mmol, 31%), then **VEcPP 5** as a white solid after lyophilization (19 mg, 0.059 mmol, 22%) and compound **15** (20 mg, 0.059 mmol, 23%).

VEcPP : ¹H NMR (D₂O, 500 MHz) : δ 3.77 (d, 1H, J_{H-H} = 12.5Hz, H_{1a}), 4.02-4.10 (m, 2H, H_{1b} and H_{4a}), 4.19-4.24 (m, 1H, H_{4b}), 4.31 (dd, 1H, J_{H-H} = 2.5Hz and 7.6Hz, H₃), 5.47 (dd, 1H, J_{H-H} = 1.4Hz and 11.0Hz, CH_aH_b vinyl), 5.59 (dd, 1H, J_{H-H} = 1.4Hz and 17.2Hz, CH_aH_b vinyl), 5.96 (ddd, 1H, J_{H-H} = 11.0 and 17.2 Hz, ³J_{P-C} = 3.6Hz, CH vinyl). ¹³C NMR (D₂O, 125 MHz) : δ 64.9 (d, ³J_{P-C} = 2.8Hz, C₁), 65.9 (d, ²J_{P-C} = 6.5Hz, C₄), 68.4 (C₃), 86.3 (d, ²J_{P-C} = 7.8Hz, Cq, C₂), 118.2 (CH₂ vinyl), 134.3 (d, ³J_{P-C} = 7.9Hz, CH vinyl). ³¹P NMR (D₂O, 202 MHz) : δ -14.9 (d, 1P, ²J_{P-P} = 23.6Hz), -11.2 (m, 1P). HRMS (ESI⁺) Calcd for C₆H₁₂O₉P₂Na (M + Na)⁺, 312.9854, Found: 312.9861. [α]_D²⁵ = -2.6° (c 0.4, MeOH).

Biological procedures

Production of *E. coli* IspG (also called GcpE): Competent cells of *E. coli* strain XL1-blue carrying the plasmid pBAD-HisGcpE^[6] were transformed with plasmid pRSF-ISC^[19] leading to the recombinant *E. coli*

XL1-blue [pBAD-GcpE, pRSF-ISC] strain. *E. coli* XL1-blue [pBAD-GcpE, pRSF-ISC] strain was grown at 37°C under 200 rpm agitation on LB medium (2×500 mL) containing ampicillin B (100 µg mL⁻¹) and kanamycin (25 µg mL⁻¹). When 0.4 OD₆₀₀ was reached, 1 mM cysteine and 100 µM FeCl₃ were then added and induction of the ISC operon was performed with 100 µM IPTG. The culture was further incubated at 30°C until reaching 0.8 OD₆₀₀. Then 0.1 % L-arabinose was added to induce the overexpression of IspG. After incubation overnight at 23°C, cells were harvested by centrifugation (6000 g, 10 min) and kept at -80°C until use.

Purification of *E. coli* IspG: The following steps were carried out in a glove box (Jacomex BS531 T2) filled with a nitrogen atmosphere containing less than 2 ppm O₂. All tubes, columns, resins, reagents and buffers were transferred in the glove box where they were left to stand several days before being utilized.

E. coli XL1-blue [pBAD-GcpE, pRSF-ISC] cell pellet (3.5 g) was resuspended in 50 mM Tris-HCl pH 8 (12 mL) containing lysozyme (1 mg mL⁻¹) and protease inhibitor cocktail (cOmplet tablets mini, Roche). After 1 h incubation at 4°C, cells were sonicated (4×15 s with 1 min cooling) then centrifuged (15000 g, 25 min) in order to collect the supernatant. DTT (1 mM), FeCl₃ (3.5 mM) and cysteine (3 mM) were added to the supernatant and the mixture stayed at 18°C for 1 h before being loaded onto a Ni²⁺-nitrilotriacetic acid agarose resin column (Qiagen, 1.5 cm × 8 cm) equilibrated with 50 mM Tris-HCl (pH 8). The resin was first washed with 50 mM Tris-HCl (pH 8) then with 30 mM imidazole in 50 mM Tris-HCl (pH 8), followed by 60 mM imidazole in 50 mM Tris-HCl (pH 8). The protein was eluted with 150 mM imidazole in 50 mM Tris-HCl (pH 8). The fractions containing the protein were pooled (> 90 % pure as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) then concentrated on Amicon Ultra 30 K (Merck Millipore) before being desalted on a PD 10 column (Cytiva) equilibrated with 50 mM Tris-HCl, pH 8. The resulting protein solution was further loaded on a HiLoad 16/200 Superdex 200 prep-grade column (GE-Healthcare) eluted with 50 mM NaCl in 50 mM Tris-HCl pH 8, in order to remove the aggregates. The protein was further concentrated on Amicon Ultra 30 K (Merck Millipore) and desalted on a PD 10 column (Cytiva) equilibrated with 50 mM Tris-HCl, pH 8. A resulting brown-green protein solution (2.3 mL, 182 µM, 18 mg) was obtained that was frozen and stored in liquid nitrogen prior to use. Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard.^[20] Iron was quantified by the method of Fish^[21] and sulphide as described by Beinert^[22] indicating 3.5 iron and over 2.5 sulphur ions per IspG molecule.

Enzymatic assays of IspG. In a typical IspG assay (150 µL final volume), a solution of IspG (12 µM final concentration) purified as described above was added using a gas-tight syringe to a 0.1 cm light path quartz cuvette prepared in an anaerobic glove box and containing NADPH (2 mM), flavodoxin (FldA, 30 µM), flavodoxin reductase (FpR1, 17 µM) and MEcPP (250 µM) in 50 mM Tris-HCl pH 8. The reaction was followed spectrophotometrically at 340 nm with a Cary 100 UV/visible spectrophotometer (Varian) kept at 37°C using a thermostat equipped with a Peltier element. A control assay was performed by replacing IspG by buffer.

To test VEcPP as a substrate of IspG, MEcPP was replaced in the IspG assay by VEcPP (500 µM).

Inhibition assays: IspG (12 µM) was added via a gas-tight syringe to a 0.1 cm light path quartz cuvette prepared in an anaerobic glove box and containing NADPH (2 mM), FldA (30 µM), FpR1 (17 µM), MEcPP (250 µM) and VEcPP (500 µM) in 50 mM Tris-HCl buffer pH 8. The reaction was followed as described above. The same assay was also performed by adding MEcPP to initiate the reaction using the same concentrations of MEcPP, IspG, VEcPP and cofactors as for the assay initiated by IspG.

Slow-binding inhibition and IC₅₀ determination: A MEcPP solution (final concentration 250 µM) was added through a gas-tight syringe to a 0.1 cm light path cuvette prepared in an anaerobic glove box and containing NADPH (2mM), FldA (30 µM), FpR1 (17 µM), IspG (12 µM) and various VEcPP concentrations (0, 50, 75, 100, 250 and 500 µM) in 50 mM Tris-HCl pH 8 that was previously incubated for 10 min or 30 min at 37°C. The reaction was monitored as described above. The initial velocity at each VEcPP concentration was plotted as dose-response curves and fit using Kaleidagraph according to the equation $y = 1/(1 + (x/IC_{50})^{slope})$ in order to determine the IC₅₀ value with y representing the fraction of inhibition and x the VEcPP concentration.

Irreversibility of VEcPP

IspG (13.5 µM) was incubated at 37 °C in an anaerobic glove box for 30 min in the presence of VEcPP (1.1 mM), NADPH (2.3 mM), FldA (33µM) and FpR1 (19 µM) in 50 mM Tris-HCl pH 8 (335 µL; medium 1). In order to verify the complete inhibition of IspG, 135 µL of medium 1 were retrieved and placed in a 0.1 cm light path quartz cuvette before MEcPP (15µL, 250 µM final concentration in the test) was added via a gas-tight syringe to initiate the reaction.

The remaining of the solution was diluted by 2 and concentrated by approximately 5 fold using a centrifugal filter (Amicon Ultra 10K, Merck Millipore). Buffer (3.5 volumes) was then added to the concentrate (1 volume) and the centrifugation-dilution cycle was pursued three more times before the final volume was adjusted to 200 µL with buffer affording medium 2. 135 µL of medium 2 was retrieved and placed in a 0.1 cm light path quartz cuvette. IspG activity was measured by adding NADPH (2 mM final concentration; as it was eliminated together with the unbound VEcPP) to the cuvette and MEcPP (250 µM) to initiate the reaction.

As no activity was detected, new quantities of FldA (26 µM) and FpR1 (16 µM) were further added to the test and their addition did not restore the IspG activity. The activity was only restored by adding to the assay a new portion of IspG (10 µM). A control sample containing no inhibitor was run under the same conditions.

Incubation of VEcPP with the reduction system in absence of IspG

NADPH (1.8 mM), FldA (36.8 µM) and FpR1 (20.9 µM) were incubated at 37 °C in an anaerobic glove box for 30 min in the presence of VEcPP (1.1 mM) in 50 mM Tris-HCl pH 8 (150 µL). The solution was diluted by 3 and concentrated by approximately 6 fold using centrifugal filters (Amicon Ultra 10K, Merck Millipore). Buffer (5 volumes) was then added to the concentrate (1 volume) and the centrifugation-dilution cycle was pursued three more times. NADPH (1.8 mM) was added before the final volume was adjusted to 150 µL with buffer. 123 µL were retrieved and placed in a 0.1 cm light path quartz cuvette together with MEcPP (15 µL at 2.5 mM). IspG activity was measured to verify that the reduction system is still active by adding IspG (12 µM) to initiate the reaction. A control sample containing no inhibitor was run under the same conditions.

Incubation of inhibited IspG with increasing amounts of MEcPP.

IspG (13.4 µM) was incubated at 37 °C in an anaerobic glove box for 30 min in the presence of NADPH (2.2 mM), FldA (33.5 µM), FpR1 (19 µM) and VEcPP (111 µM) in 50 mM Tris-HCl pH 8 (860 µL).

The solution was adjusted to 1 mL and concentrated by approximately 5 fold using a centrifugal filter (Amicon Ultra 10K, Merck Millipore). Buffer (4 volumes) was then added to the concentrate (1 volume) and the centrifugation-dilution cycle was pursued three more times. NADPH (2.2 mM) was added before the final volume was adjusted to 860 µL with buffer.

135 µL of the solution were retrieved and placed in a 0.1 cm light path quartz cuvette. IspG activity was measured by adding various concentrations of MEcPP to initiate the reaction.

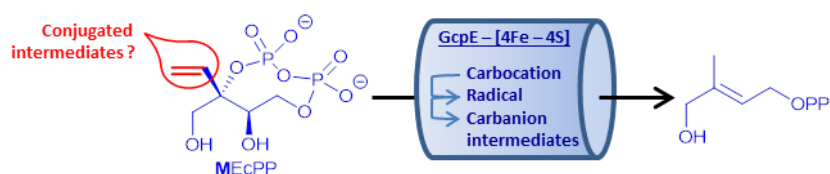
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Synthesis of a substrate analogue of MEcPP, a key intermediate in the MEP pathway, has been achieved. Target compound features a vinyl functionality in place of the natural methyl substituent. Potent irreversible inhibition was observed towards GcpE (IspG). Introduction of the double bond allows conjugation in the putative reactive intermediates while entering enzymatic transformation. Unexpected covalent trapping could happen as consequential damage.