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Disruption of insect isoprenoid biosynthesis with pyridinium bisphosphonates

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ABSTRACT

Farnesyl diphosphate synthase (FPPS) catalyzes the condensation of the non-allylic diphosphate, isopentenyl diphosphate (IPP; C_5), with the allylic diphosphate primer dimethylallyl diphosphate (DMAPP; C_5) to generate the C_{15} prenyl chain (FPP) used for protein prenylation as well as sterol and terpene biosynthesis. Here, we designed and prepared a series of pyridinium bisphosphonate (PyrBP) compounds, with the aim of selectively inhibiting FPPS of the lepidopteran insect order. FPPSs of *Drosophila melanogaster* and the spruce budworm, *Choristoneura fumiferana*, were inhibited by several PyrBPs, and as hypothesized, larger bisphosphonates were more selective for the lepidopteran protein and completely inactive towards dipteran and vertebrate FPPSs. Cell growth of a *D. melanogaster* cell line was adversely affected by exposure to PyrPBs that were strongly inhibitory to insect FPPS, although their effect was less pronounced than that observed upon exposure to the electron transport disrupter, chlorfenapyr. To assess the impact of PyrBPs on lepidopteran insect growth and development, we performed feeding and topical studies, using the tobacco hornworm, *Manduca sexta*, as our insect model. The free acid form of a PyrBP and a known bisphosphonate inhibitor of vertebrate FPPS, alendronate, had little to no effect on larval *M. sexta*; however, the topical application of more lipophilic ester PyrBPs caused decreased growth, incomplete larval molting, cuticle darkening at the site of application, and for those insects that survived, the formation of larval–pupal hybrids. To gain a better understanding of the structural differences that produce selective lepidopteran FPPS inhibition, homology models of *C. fumiferana* and *D. melanogaster* FPPS (*Cf*FPPS2, and *Dm*FPPS) were prepared. Docking of substrates and PyrBPs demonstrates that differences at the −3 and −4 positions relative to the first aspartate rich motif (FARM) are important factors in the ability of the lepidopteran enzyme to produce homologous isoprenoid structure and to be selectively inhibited by larger PyrBPs.

1. Introduction

Isoprenoids are ubiquitous in nature and serve varied roles in cellular metabolism and signaling. The construction of compounds with carbon skeletons larger than monoterpenes requires the intermediacy of the enzyme farnesyl diphosphate synthase (FPPS), which catalyzes the head to tail condensation of the allylic primers dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) with the product of the mevalonate and DOX pathways, isopentenyl diphophate (IPP, Dhar, et al., 2013). The importance of FPPS in cellular function can be seen by its requirement for the construction of essential natural products, its role in protein prenylation, and its regulation of the mevalonate pathway (Szkopinska et al., 2000). A significant number of proteins undergo post-translational modification through farnesyl- or geranylgeranylation, which induces their association to membranes or their interaction with other proteins (Palsuledesai and Distefano, 2015; Resh 2013, Rilling et al., 1990). Mutations in several gene products, including proteins of the *Ras* family, have been linked to cancers and because of this, FPPS has been targeted for the development of anti-cancer agents (Agrawal and Somani, 2009).

A specific group of FPPS inhibitors, the bisphosphonates (BPs),

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have found widespread use as chemotherapeutics against bone disease (Xu et al., 2013). The bisphosphonate moiety serves as a mimic of the diphosphate group that is present in the allylic diphosphate substrate, and when tethered to a nitrogen-containing group, the resulting N-BP can function as a transition state analog, mimicking the ion pair that is produced during catalysis by FPPS. Because BPs form strong chelates with calcium ions, N-BPs are accumulated in bone where they are able to target FPPS of localized cells (Roelofs et al., 2010). N-BPs act directly on osteoclasts and indirectly on osteoblasts and as a result, they are used clinically for the treatment and prevention of osteoporosis, and in the treatment of hypercalcemia, tumor-induced osteolysis, and Paget's disease. Bisphosphonates have been utilized as novel inhibitors of FPPS in several parasitic organisms, including *Typanosoma cruzi* (the causative agent of Chagas disease), *Toxoplasma gondii* (the causative agent of toxoplasmosis), *Plasmodium falciparum* (the causative agent of malaria), and *Entamoeba histolytica* (the causative agent of amebic dysentery, Gabelli et al., 2006; Ling et al., 2007; Srivastava et al., 2008).

As with other organisms, insects produce and utilize isoprenoids for a wide range of biochemical processes (Hick et al., 1999; Gershenzon and Dudareva, 2007). In contrast to other animals and more similarly to plants, insects produce a wider range of isoprenoid structures including monoterpenes (e.g., aggregation and sex pheromones), sesquiterpenes (e.g., juvenile hormone, JH), and diterpenes (e.g., pheromonal cembrenes). While protein prenylation in insects is not well understood, this process appears to be important for insect function and development. In *Drosophila melanogaster* protein prenylation was shown to be essential for the phosphodiesterase, PDE5/6, inducing its localization to the membrane where it plays an important role in cyclic guanosine monophosphate (cGMP) transport in Malpighian tubules (Day et al., 2008). Disruption of the FPPS gene in *D. melanogaster* is lethal, causing a significant decrease in embryonic crystal cell formation as a result of abolished protein farnesylation (Milchanowski et al., 2004).

A unique feature of insects of the lepidopteran order is their ability to produce homologous JHs, which are utilized as analogs of the ubiquitous JH III (Bergot et al., 1981). The biosynthesis of these structures is due to the incorporation of propionyl-CoA into the mevalonate pathway (Brindle et al., 1988) and the presence of an FPPS that is readily able to couple homologous diphosphate substrates (Koyama et al., 1985). Consistent with the unique features of lepidopteran JH biosynthesis is the presence of two distinct FPPS paralogs (termed type-1 and type-2) in Lepidoptera (Cusson et al., 2006; Kinjoh et al., 2007; Sen et al., 2007a,b). Type-1 FPPSs were observed to display several active site substitutions when compared to “conventional” eukaryotic FPPSs, particularly in the first aspartate rich motif (FARM) region, where NDxxE substitutes for DDxxD. In comparison, the type-2 (FPPS2) protein displays a more conventional active site. Although type-1 (FPPS1) at first appeared be the better candidate for binding and coupling homologous substrates, transcriptional analysis of both FPPS1 and FPPS2 in various *Bombyx mori* (Cusson et al., 2006; Kinjo et al., 2007) and *Pseudaletia unipuncta* (Barbar, 2014) tissues revealed a confinement to the CA for the latter. This observation led to the conclusion that FPPS2 was likely the principal prenyltransferase generating FPP substrates for JH biosynthesis.

Using homogenates of *Manduca sexta* corpora allata (the exclusive site of JH carbon skeleton biosynthesis), we demonstrated that the substrate specificity of lepidopteran FPPS has exquisitely evolved to readily accommodate homologous allylic diphosphate substrates (Sen et al. 1996, 2007a,b). Thus, dimethylallyl diphosphate analogs with C-3 methyl substitution by R groups ranging from ethyl (C2) to *n*-butyl (C4) bound the lepidopteran FPPS, as indicated by competitive inhibition, and analogs with R groups as large as *n*-propyl (C3) were coupled to IPP, indicating their ability to serve as alternate substrates. In comparison, vertebrate FPPS showed a linear decrease in its capacity to accommodate larger DMAPP analogs. The natural substrate of lepidopteran JH homolog biosynthesis, homodimethylallyl diphosphate (HDMAPP) was a significantly worse substrate of vertebrate FPPS (Nishino et al., 1972, 1973; Sen et al., 2006) and the *n*-propyl substrate was not coupled to IPP. The involvement of lepidopteran FPPS2 in these reactions was demonstrated by identification of corpora allata *M. sexta* FPPS as a type-2 paralog and with the expression and purification of *Choristoneura fumiferana* FPPS2 (*Cf*FPPS2) which readily coupled homologous dimethylallyl and geranyl diphosphates with IPP (Sen et al., 2007b).

FPPS has been studied as a potential bio-rational target site for insecticide development because of its pivotal role in the biosynthesis of JH (Cusson et al., 2012) and its unique structural and catalytic features in certain groups of insects (Cusson et al., 2006; Vandermoten et al., 2008). The Lepidoptera include a large number of agricultural pests (Stevens et al., 2012), and as lepidopteran JH biosynthesis displays biochemical features that are unique to this insect order, inhibition of lepidopteran FPPS may show promise for the development of order-specific insecticides. Beyond this, FPPS paralogs appear to be involved in other important pathways besides JH biosynthesis; therefore, their inhibition could result in multiple physiological disturbances that would compromise the proper physiology of the insect.

Here, we report on the design, synthesis and evaluation of several pyridinium bisphosphonates (PyrBPs) as inhibitors of isoprenoid biosynthesis and as selective inhibitors of lepidopteran FPPS2. The ability of PyrBPs to affect the viability of insect cells and ultimately animal growth and development has also been demonstrated. This first detailed study suggests a new avenue for insecticide development, utilizing a rational and site-directed approach, based on insect FPPS structure and function.

2. Materials and methods

2.1. Chemical sources and insects

[^{14}C]IPP (specific activity 59 mCi/mmol) was obtained from Amersham-Pharmacia Biotech, UK. All other fine chemicals and reagents were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). *Manduca sexta* larvae were reared on artificial diet (Bell and Joachim, 1976) at 26 °C, in a humidity-controlled incubator set on a 18:6 h light cycle, from eggs purchased from Great Lakes Hornworm (Troy, MI).

2.2. Synthesis of pyridinium bisphosphonates for inhibitor studies

Pyridinium bisphosphonates **3a**–**3e** were prepared as described in Scheme 1. Briefly, 2-(bromomethyl)pyridine was reacted with the sodium anion of tetramethyl- or tetraethyl methylene bisphosphonate to produce bisphosphonate esters **1** and **2**, respectively. Alklyation of **2** produced N-methyl (**2b**), N-ethyl (**2c**), N-propyl (**2d**), and N-butyl (**2e**) derivatives, which were then converted (along with **1**) to the corresponding bisphosphonic acids by treatment with 6 N aqueous HCl.

2.2.1. Tetramethyl [2-(2-pyridyl)ethyl]-1,1-bisphosphonate (*1*)

To a suspension of sodium hydride (395 mg, 10 mmol) in 20 mL of dry THF under argon was added 15-crown-5 (2 mL, 10 mmol), followed by the dropwise addition of tetramethyl methylene bisphosphonate (2.4 mL, 12 mmol). While the reaction mixture stirred at rt for 0.5 h, 2-(bromomethyl)pyridine hydrobromide