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Synthesis and biological evaluation of 1-amino-1,1-bisphosphonates derived from fatty acids against *Trypanosoma cruzi* targeting farnesyl pyrophosphate synthase

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Abstract—We have investigated the effect of a series of 1-amino-1,1-bisphosphonates derived from fatty acids against proliferation of the clinically more relevant form of *Trypanosoma cruzi*, the causative agent of American trypanosomiasis (Chagas' disease). Some of these drugs were potent inhibitors against the intracellular form of the parasite, exhibiting IC_{50} values at low micromolar level. Cellular activity was associated with the inhibition of enzymatic activity of *T. cruzi* farnesyl pyrophosphate synthase. As bisphosphonate-containing drugs are FDA-approved for the treatment of bone resorption disorders, their potential innocuousness makes them good candidates to control tropical diseases.

Trypanosoma cruzi is the hemoflagellated protozoan parasite that causes American trypanosomiasis (Chagas' disease), which is an endemic disease widespread from southern United States to southern Argentina. It has been estimated that around 18 million people are infected and over 40 million are at risk of infection by *T. cruzi*.[1] This disease is considered by the World Health Organization to be one of the major parasitic diseases. Like other kinetoplastid parasites, *T. cruzi* has a complex life cycle possessing three main morphological forms: it multiplies in the insect gut in the non-infective epimastigote form and is spread as a non-dividing metacyclic trypomastigote from the insect feces by contamination of intact mucosa or wounds produced by the blood-sucking activity of the vector (Reduviid insect). In the mammalian host, *T. cruzi* multiplies intracellularly in the amastigote form and is subsequently released into the bloodstream as a non-dividing trypomastigote.[2] Transmission of Chagas' disease could also occur via the placenta or by blood transfusion.[3] This latter mechanism is responsible for the occurrence of Chagas' disease in developed countries where the disease is not endemic.[3,4]

Chagas' disease goes through an acute phase, which may happen virtually unnoticed, although infrequently it can lead to fatal meningoencephalitis or acute myocarditis, mostly in adults; an indeterminate asymptomatic phase, which can persist for more than 10 years or even for the entire life of an infected individual; and finally, a chronic phase, associated with heart problems or enlargement of hollow viscera (esophagus and colon) that may lead to death. Chemotherapy for the treatment of Chagas' disease, which is based on two empirically discovered drugs, nifurtimox, now discontinued, and benznidazole, is still deficient.[1,5,6] Although both these compounds are able to cure at least 50% of recent infections, they suffer from major drawbacks, such as: (a) selective drug sensitivity on different *T. cruzi* strains; (b) serious side effects including vomiting, anorexia, peripheral neuropathy, allergic dermopathy, etc.; (c) long-term treatment.[1] Moreover, these compounds are not effective during the chronic stage of the disease. In addition, there are a number of uncertainties pertaining to gentian violet, the only drug available to prevent transmission of Chagas' disease through blood, as it is carcinogenic to

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animals.[7] On account of the above-mentioned reasons, there is a critical need to develop new drugs that are more effective and safer than those presently available.[1,7] The knowledge of unique features of the biochemistry and physiology of *T. cruzi* has led to the identification of specific molecular targets for rational drug design.[8–13] Farnesyl pyrophosphate synthase proved to be a convenient target for many tropical diseases.[12,13]

Bisphosphonate derivatives, such as pamidronate (**1**), alendronate (**2**), and risedronate (**3**) and other bisphosphonates, which are effective inhibitors of bone resorption, have also been found to be effective growth inhibitors of pathogenic trypanosomatids (*T. cruzi*, *T. brucei rhodesiense*, *Leishmania donovani*, and *L. mexicana*) and apicomplexan parasites (*Toxoplasma gondii* and *Plasmodium falciparum*).[14–20] In addition, in vivo studies of risedronate indicated that this drug exhibits a selective antiproliferative activity against *T. cruzi* in a murine model of acute Chagas' disease. These results reinforce the potential utility of bisphosphonates as specific chemotherapeutic agents against Chagas' disease.[21] The chemical structures of these representative bisphosphonates are illustrated in Figure 1. Bisphosphonates are FDA-approved drugs for the treatment of several bone disorders including osteoporosis, Paget's disease, problems associated with bone metastases and multiple myeloma, hypercalcemia provoked by malignancy, and bone inflammation associated with periodontal disease or rheumatoid arthritis.[22–25] Geminal phosphonates are isosteric analogues of inorganic pyrophosphate in which a methylene group has replaced the oxygen bridge between the phosphorus atoms. Unlike pyrophosphates, bisphosphonates possess better metabolic stability because they are not recognized by pyrophosphatases and are also stable to hydrolysis under acidic media.

The exact mode of action of bisphosphonates at the isoprenoid pathway has been recently elucidated, regardless of having been used for more than 30 years as an effective class of anti-resorptive drugs.[26,27] The molecular target is farnesyl pyrophosphate synthase (FPPS), an enzyme that catalyzes the formation of the substrate for protein prenylation. Previous studies have postulated that these bisphosphonates were putative inhibitors of pyrophosphate-related metabolic pathways. Actually, trypanosomatids require protein prenylation for survival,[28] a process that is responsible for the attachment of farnesyl and geranylgeranyl groups to the C-terminal cysteine residues of a number of GTPase signaling proteins, giving rise to farnesylated and geranylgeranylated proteins. These transfer reactions are catalyzed at least by three different cytoplasmic prenyl protein transferases.[29] These proteins are important signaling molecules involved in crucial cell processes for osteoclast function.[27] The attached farnesyl and geranylgeranyl groups seem to be crucial for anchoring proteins to membranes and consequently their biological action.[29] Selective inhibition of prenyl protein transferases slows proliferation of human tumors due to farnesylation inhibition of oncogenic Ras.[30] On the basis of this result, many prenyl protein transferase inhibitors have been developed, which resulted not only in potential antitumor agents,[30] but also potent growth inhibitors of *T. cruzi* and *T. brucei*.[28] Bearing in mind that bisphosphonates are FDA-approved drugs for long-term treatment of bone disorders, low toxicity for bisphosphonate-containing drugs might be predictable.

Most pharmacologically important bisphosphonates are nitrogen-containing drugs. It had been postulated that the presence of a nitrogen atom at the C-3 position would act as carbocation transition state analogue of isoprenoid diphosphates for isoprenoid biosynthesis.[31,32] Of special interest are bisphosphonates derived from fatty acids, in which no nitrogen atom is present in their chemical structure.[18–20] These compounds were shown to be potent growth inhibitors against the clinically more relevant form of *T. cruzi* bearing IC_{50} values at the low micromolar level.[19,20] Compound **4** arises as the main member of this new family of drugs with an IC_{50} value of 18 μM. This cellular activity correlates quite properly with the inhibition of the enzymatic activity toward *Tc*FPPS,[20] being a competitive inhibitor with an IC_{50} value of 1.94 μM and a K_i of 0.40 μM.[20] Compound **4** also inhibits the enzymatic activity of *T. brucei* FPPS[20] and is active in vitro against *T. gondii*.[18] Since this class of drugs are devoid of a nitrogen atom, they cannot act as carbocation transition state analogues of the substrate. By this time, it is known that the isosteric replacement of the hydroxyl group at C-1 by a hydrogen atom impairs a bisphosphonate potency against amastigotes (see Fig. 2).

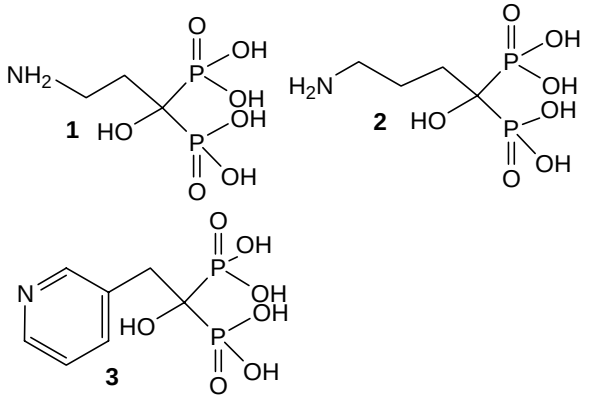


Figure 1. Chemical structures of a representative member of bisphosphonates currently employed for the treatment of bone disorders.

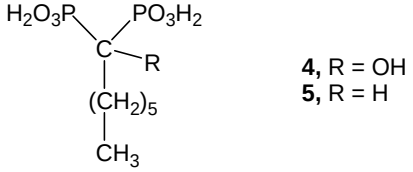


Figure 2. Chemical structures of representative bisphosphonates derived from fatty acids.

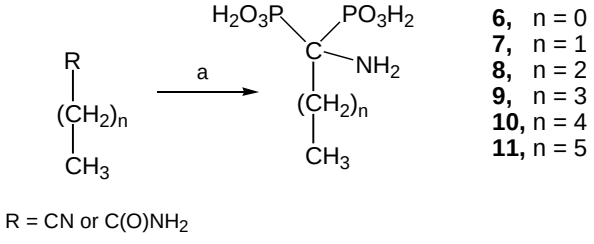
Taking **5** as an example, the cellular activity of this drug has an IC_{50} value close to 70 μM and a high efficacy to inhibit the enzymatic activity of *Tc*FPPS (IC_{50} = 4.54 μM, and K_i of 0.54 μM, respectively).[20]

On the basis of potent inhibitory action exhibited by 1-hydroxy-1,1-bisphosphonates derived from fatty acids, it was decided to conduct further studies on the influence of the hydroxyl group at the C-1 position on their biological activity. Taking **5** as an example, low efficacy observed for this type of drugs in which the hydroxyl group at C-1 is missing may be attributed to the lack of ability to coordinate Mg^{2+} present at the active site of the target enzyme.[33,34] Although this capability plays an important role in biological activity, the fact that compounds **4** and **5** exhibited similar K_i values indicates that other factors might have influence either on enzymatic molecular recognition or on biological activity against *T. cruzi* cells. For the above reasons, the isosteric replacement of the hydroxyl group by an amino unit at carbon-1 as a structural variation seemed to be of interest. The presence of an amino group retains the ability to coordinate Mg^{2+} in a tridentate manner similar to the 1-hydroxy-1,1-bisphosphonate derivatives.

The compounds could be prepared starting either from the corresponding cyano derivatives[35,36] or from the respective amides.[37,38] In the present study, all the designed drugs were prepared from the appropriate nitrile, with the exception of compound **11**, which was synthesized starting from *n*-heptanamide. The preparation of this new family of *n*-alkyl 1-amino *gem*-bisphosphonates is given in Scheme 1. All compounds were routinely characterized by using ^1H, ^{13}C, and ^{31}P NMR spectroscopy at 500, 125, and 242 MHz, respectively (Bruker AM-500 apparatus). Elemental analysis data for all new compounds were satisfactory.[39] These compounds were evaluated as growth inhibitors against *T. cruzi* (epimastigotes and amastigotes). In addition, correlation of the cellular activity with the action against its target enzyme was studied. WC-9, a well-known antiparasitic agent, was used as a positive control.[40,41]

1-Amino-1,1-bisphosphonates derived from fatty acids (compounds **6**–**11**) were potent inhibitors of *Tc*FPPS activity. The 1-amino derivatives were even more effective than the 1-hydroxy-1,1-bisphosphonate derivatives toward *Tc*FPPS (Table 1).[20] Once again, the efficacy of each drug on this enzyme qualitatively correlated with the inhibitory action that exhibited against *T. cruzi* (amastigotes) growth. Taking compound **10** as an example, this compound was a potent inhibitor of *Tc*FPPS activity with an IC_{50} in the nanomolar range (0.38 μM). These data correlated completely with the efficacy of this drug as an antiparasitic agent. In fact, compound **10** proved to be a potent inhibitor of the clinically more relevant form of the parasite with an IC_{50} = 77 μM, but to a slightly lesser extent than the effectiveness previously observed by drug **4** (IC_{50} = 18.0 μM) taken as lead drug.[20] A comparable degree of efficacy as inhibitors of *Tc*FPPS activity was observed for compounds **9** and **11**. Surprisingly, in spite of being one order of magnitude more potent than **11** towards FPPS, drug **10** exhibited an indistinguishable antiproliferative potency against amastigotes compared to compound **11**. This unexpected behavior may be attributed to the fact that these compounds would present different pharmacokinetic aspects. Short alkyl chain 1-amino-1,1-bisphosphonates exhibited marginal activity not only as enzymatic inhibitors, but also as growth inhibitors against *T. cruzi* (amastigotes). Compounds **6**–**11** lacked activity against the non-infective epimastigote forms of the parasite. This behavior was in agreement with the activity that had been observed for bisphosphonates derived from fatty acids (compounds of general formula **4** and **5**, that is, with a hydroxyl group at C-1, or replacing it by a hydrogen atom, respectively). The results are shown in Table 1. The activity of the enzyme *Tc*FPPS was measured by a radiometric assay based on that depicted before.[42–44]

It has been found that FPPS requires certain concentration of Mg^{2+} for optimal activity.[31,42] In fact, crystal structure of this protein indicates that two or three Mg^{2+} ions can be present at the active site. This behavior can be observed in protein structures (PDB files 1FPS, 1UBV, 1UBW, 1UBX, 1UBY, 1YQ7, and 1YV5).[32,45,46]



Scheme 1. Reagents and conditions: (a) MeCN, H_3PO_3, 130 °C, 12 h, 51% for **6**; CH_3CH_2CN, H_3PO_3, 135 °C, 12 h, 70% for **7**; (i) $CH_3CH_2CH_2CN$, H_3PO_3, $PhSO_3H$, 65 °C, 10 min, (ii) PCl_3, 70 °C, 6 h, 53% for **8**; (i) $CH_3(CH_2)_3CN$, H_3PO_3, $PhSO_3H$, 65 °C, 10 min, (ii) PCl_3, 85 °C, 16 h, 25% for **9**; (i) $CH_3(CH_2)_4CN$, H_3PO_3, $PhSO_3H$, 70 °C, 10 min, (ii) PCl_3, 90 °C, 16 h, 15% for **10**; (i) $CH_3(CH_2)_4CH_2C(O)NH_2$, H_3PO_3, $PhSO_3H$, 65 °C, 10 min, (ii) PCl_3, 90 °C, 16 h, 10% for **11**.

Table 1. Effect of alkyl 1-amino-1,1-bisphosphonates toward *T. cruzi* farnesyl pyrophosphate synthase activity and against *T. cruzi* (amastigotes and epimastigotes) growth for compounds **6**–**11**

Compound	IC_{50} (μM)[a,b]		
	*Tc*FPPS	Epimastigotes	Amastigotes
6	66.65 ± 3.39	>100.0	>100 μM
7	149.59 ± 30.09	>100.0	>85 (13%)
8	30.77 ± 3.01	>100.0	>85 (13%)
9	3.91 ± 0.58	>100.0	>85 (32%)
10	0.382 ± 0.039	>100.0	77.0
11	3.57 ± 0.28	>100.0	72.0
WC-9	—	2.2[19]	16.0[19]

[a] Values are means of three experiments. IC_{50} values for FPPS were calculated as described.[20]

[b] Maximum inhibition values obtained at the indicated concentrations (ca. 85.0 μM).

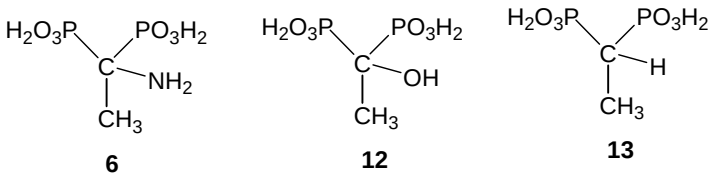


Figure 3. Selected chemical structures of 1-amino-, 1-hydroxy-, and alkyl-1,1-bisphosphonates to study their ability to coordinate Mg^{2+}.

In addition, the ability of representative bisphosphonates to coordinate different ions, such as Cu^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Al^{3+}, etc., has been studied by means of NMR spectroscopy.[47–49] It was considered of interest to study the capability of compounds **6**, **12**, and **13** to coordinate Mg^{2+} (Fig. 3). These drugs were selected on account of their large solubility in water. All these compounds had three different groups at C-1, amino, hydroxy, and a hydrogen atom, and also the same chain length, regardless of the inhibitory potency. The results were very encouraging. Therefore, the respective ^{31}P NMR spectrum for each compound was recorded versus increasing concentration of magnesium chloride. Analysis of the ^{31}P NMR spectra points out that the chemical shift moves upfield as the concentration of Mg^{2+} ion increases. This effect was more noticeable for **6** and **12**, and to a lesser extent for **13**. These results indicated that **6** and **12** coordinated stronger with Mg^{2+} than **13** and are in agreement with the modest efficacy observed when the hydrogen atom is the substituent at C-1. This behavior was expected because **6** and **12** coordinate ions in a tridentate manner. ^{31}P chemical shift of these compounds was not affected by the medium ionic strength (see Fig. 4).

It can be concluded that 1-amino-1,1-bisphosphonates derived from fatty acids were potent inhibitors of *Tc*FPPS. Compound **10** was able to inhibit the activity of this enzyme at the nanomolar range and even more potent than any of the 1-hydroxy-1,1-bisphosphonates previously tested toward *Tc*FPPS.[20] In spite of the inhibitory action against *Tc*FPPS, cellular activity of **10** was not more efficient than the 1-hydroxy derivatives, probably due to its pharmacokinetic properties.

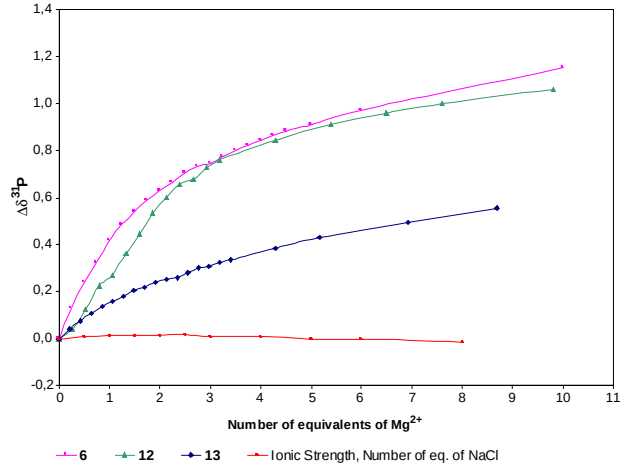


Figure 4. Effect of the concentration of Mg^{2+} ion on the ^{31}P chemical shift for compounds **6**, **12**, and **13**.

Work aimed at optimizing the chemical structure of compounds, such as **4** and **10**, as well as in establishing a thorough structure–activity relationship, is currently being pursued in our laboratory.

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39. Compound **6**: A mixture of H_3PO_3 (4.1 g, 50 mmol) and acetonitrile (1.0 mL, 20 mmol) was stirred at 130 °C for 12 h under argon atmosphere. Methanol (10 mL) was added and the solid was filtered and crystallized from water–methanol to afford 2.10 g (51% yield) of pure compound **6** as a white solid: mp 266–269 °C; ^{1}H NMR (500.13 MHz, D_2O) δ 1.54 (t, J = 14.2 Hz, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 18.28 (t, J = 3.0 Hz, C-2), 54.39 (t, J = 131.8 Hz, C-1); ^{31}P NMR (202.45 MHz, D_2O) δ 13.36. Anal. Calcd for $C_2H_9O_6P_2N$: C, 11.72; H, 4.42; N, 6.83. Found: C, 11.63; H, 4.55; N, 6.73. Compound **7**: SB13 A mixture of H_3PO_3 (3.57 g, 43.5 mmol) and propionitrile (0.77 mL, 10.8 mmol) was stirred at 135 °C for 12 h under argon atmosphere. Methanol (10 mL) was added and the solid was filtered and crystallized from 1.0 N HCl–ethanol to afford 1.67 g (70% yield) of pure compound **7** as a white solid: mp 250–251 °C; ^{1}H NMR (D_2O) δ 0.82 (t, J = 7.6 Hz, 3H, H-3), 1.65 (tq, J = 13.5, 7.4 Hz, 2H, H-2); ^{13}C NMR (D_2O) δ 10.43 (t, J = 6.7 Hz, C-2), 29.39 (C-3), 57.51 (t, J = 127.6 Hz, C-1); ^{31}P NMR (D_2O) δ 20.89. Anal. Calcd for $C_3H_{11}O_6P_2N$: C, 16.45; H, 5.06; N, 6.39. Found: C, 16.50; H, 5.00; N, 6.33. Compound **8**: A mixture of butyronitrile (1.26 mL, 11.3 mmol), H_3PO_3 (1.86 g, 22.7 mmol), and anhydrous benzenesulfonic acid (10 g) was stirred at 65 °C for 10 min under argon atmosphere: Then, PCl_3 (1.14 mL, 11.3 mmol) was added dropwise with vigorous stirring. The reaction was stirred at 70 °C for 6 h. Water (40 mL) was added and the mixture was stirred at room temperature for 1 h. Ethanol (20 mL) was added and the resulting product was filtered and crystallized from (c) HCl–ethanol to afford 1.77 g (53% yield) of pure compound **8** as a white solid: mp 255–257 °C; ^{1}H NMR (D_2O) δ 0.77 (t, J = 7.3 Hz, 3H, H-4), 1.29–1.49 (m, 2H, H-3), 1.73–1.95 (m, 2H, H-2); ^{13}C NMR (D_2O) δ 12.28 (C-4), 15.50 (t, J = 5.4 Hz, C-2), 31.25 (C-3), 55.26 (t, J = 127.7 Hz, C-1); ^{31}P NMR (D_2O) δ 20.94. Anal. Calcd for $C_4H_{13}O_6P_2N$: C, 20.61; H, 5.62; N, 6.01. Found: C, 20.70; H, 5.64; N, 6.05. Compound **9**: A mixture of valeronitrile (1.26 mL, 11.3 mmol), H_3PO_3 (1.86 g, 22.7 mmol), and anhydrous benzenesulfonic acid (10 g) was stirred at 65 °C for 10 min under argon atmosphere. Then, PCl_3 (1.14 mL, 11.3 mmol) was added dropwise and the reaction mixture was stirred at 85 °C for 16 h. The reaction was allowed to cool to room temperature. Water (40 mL) was added and the mixture was stirred for 10 min more. Ethanol (20 mL) was added, and the resulting product was filtered and crystallized from (c) HCl–ethanol to afford 720 mg (25% yield) of pure compound **9** as a white solid: mp 245–247 °C; ^{1}H NMR (KOD 40% (w/w) in D_2O) δ 0.77 (t, J = 7.4 Hz, 3H, H-5), 1.08–1.15 (m, 2H, H-4), 1.26–1.32 (m, 2H, H-3), 1.47–1.55 (m, 2H, H-2); ^{13}C NMR (40% KOD (w/w) in D_2O) δ 14.39 (C-5), 24.24 (C-4), 27.53 (t, J = 5.4 Hz, C-2), 36.90 (C-3), 57.26 (t, J = 128.2 Hz, C-1); ^{31}P NMR (KOD 40% (w/w) in D_2O) δ 20.82 (s); Anal. Calcd for $C_5H_{15}O_6P_2N$: C, 24.30; H, 6.12; N, 5.67. Found: C, 24.42; H, 6.13; N, 5.67. Compound **10**: A mixture of hexanenitrile (1.25 mL, 10 mmol), H_3PO_3 (1.68 g, 20 mmol), and anhydrous benzenesulfonic acid (10 g) was heated at 65 °C under argon atmosphere. Then, PCl_3 (1.0 mL, 10 mmol) was added dropwise with vigorous stirring. The reaction mixture was stirred at 90 °C for 16 h. Water (40 mL) was added and the reaction mixture was stirred a room temperature for 1 h. Ethanol (20 mL) was added, and the resulting product was filtered and crystallized from (c) HCl–ethanol to afford 397 mg (15% yield) of pure compound **10** as a white solid: mp 240–242 °C; ^{1}H NMR (40% KOD (w/w) in D_2O) δ 0.64 (t, J = 7.6 Hz, 3H, H-6), 0.98–1.02 (m, 2H, H-5), 1.05–1.10 (m, 2H, H-4), 1.22–1.28 (m, 2H, H-3), 1.48–1.57 (m, 2H, H-2); ^{13}C NMR (40% KOD (w/w) in D_2O) δ 14.50 (C-6), 22.96 (C-5), 25.09 (t, J = 6.0 Hz, C-2); 33.56 (C-3), 37.11 (C-4), 57.34 (t, J = 127.6 Hz, C-1); ^{31}P NMR (40% KOD (w/w) in D_2O) δ 20.95. Anal. Calcd for $C_6H_{17}O_6P_2N$: C, 27.59; H, 6.56; N, 5.36. Found: C, 27.78; H, 6.64; N, 5.41. Compound **11**: A mixture of heptanoamide (800 mg, 6.2 mmol), H_3PO_3 (508 mg, 6.2 mmol), and anhydrous benzenesulfonic acid (10 g) was stirred at 70 °C under argon atmosphere for 10 min. Then, PCl_3 (1.86 mL, 18.6 mmol) was added dropwise and the reaction mixture was stirred at 90 °C for 16 h. The reaction mixture was allowed to cool to room temperature. Water (40 mL) was added and the mixture was stirred at room temperature for 2 h. Ethanol (20 mL) was added, and the resulting mixture was kept at

0 °C for 24 h. The solid was filtered and crystallized from water–ethanol to afford 170 mg (10% yield) of pure **11** as a white solid: mp 246–248 °C; ^{13}C NMR (40% KOD (w/w) in D_2O) δ 14.30 (C-7), 23.09 (C-6), 25.52 (t, J = 6.7 Hz, C-2), 31.07 (C-3), 32.24 (C-4), 37.18 (C-5), 57.54 (t, J = 124.3 Hz, C-1); ^{31}P NMR (KOD 40% (w/w) in D_2O) δ 20.96. Anal. Calcd for $C_7H_{19}O_6P_2N$: C, 30.55; H, 6.96; N, 5.09. Found: C, 30.65; H, 6.99; N, 5.13.

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44. *FPPS assay and product analysis*. Briefly, 100 μL of assay buffer (10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 47 μM [4-^{14}C]IPP (10 μCi/μmol)), and 55 μm DMAPP or GPP was prewarmed to 37 °C. The assay was initiated by the addition of recombinant protein (10–20 ng), allowed to proceed for 30 min at 37 °C, and quenched by the addition of 6 M HCl (10 μL). The reactions were made alkaline with 6.0 M NaOH (15 μL), diluted in water (0.7 mL), and extracted with hexane (1 mL). The hexane solution was washed with water and transferred to a scintillation vial for counting. One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [4-^{14}C]IPP into [14-^{14}C]FPP in 1 min.
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