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Synthesis of ATP derivatives of compounds of the mevalonate pathway (isopentenyl di- and triphosphate; geranyl di- and triphosphate, farnesyl di- and triphosphate, and dimethylallyl diphosphate) catalyzed by T4 RNA ligase, T4 DNA ligase and other ligases. Potential relationship with the effect of bisphosphonates on osteoclasts

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Synthesis of ATP derivatives of compounds of the mevalonate pathway (isopentenyl di- and triphosphate; geranyl di- and triphosphate, farnesyl di- and triphosphate, and dimethylallyl diphosphate) catalyzed by T4 RNA ligase, T4 DNA ligase and other ligases. Potential relationship with the effect of bisphosphonates on osteoclasts.

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Abbreviations: Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetrphosphate; Apppl or iso-pppA, ATP derivative of iso-PP; BP, a bisphosphonate; dim-PP, dimethylallyl diphosphate; dim-pppA, ATP derivative of dim-PP; far-PP or far-PPP, farnesyl di- or triphosphate; ger-PP or ger-PPP, geranyl di- or triphosphate; iso-PP or iso-PPP, isopentenyl di- or triphosphate; iso-pppA or iso-ppppA, ATP derivative of iso-PP or iso-PPP; mev-PP or mev-PPP, compounds of the mevalonate pathway containing two or three terminal phosphates; mev-pppA or mev-ppppA, ATP derivatives of mev-PP or mev-PPP; N-BP, a bisphosphonate containing nitrogen; non-N-BP, a bisphosphonate not containing nitrogen; p₄A, adenosine 5'-tetrphosphate.

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ABSTRACT

1
2 Compounds of the mevalonate pathway containing a terminal di- or
3
4 triphosphate (mev-PP or mev-PPP) were tested as substrates of several
5
6 enzyme ligases (T4 RNA ligase, T4 DNA ligase, firefly luciferase and other
7
8 ligases) for the synthesis of ATP derivatives of the mev-pppA or mev-ppppA
9
10 type. T4 RNA ligase, in the presence of ATP and the substrates: geranyl,
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12 farnesyl or isopentenyl triphosphates, and geranyl, farnesyl, dimethylallyl or
13
14 isopentenyl diphosphates, all at 0.3 mM concentration, catalyzed the
15
16 synthesis of the corresponding ATP derivatives at a relative rate of activity
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18 of: 7.6 ± 1.4 mU/mg or 100 %; 39%; 42%; 24%; 18%; 12% and 6%,
19
20 respectively. Inhibition (%) of the synthesis by excess of substrate (0.8 mM
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22 vs. 0.3 mM) was observed with farnesyl diphosphate (99%); farnesyl
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24 triphosphate (96%) and geranyl triphosphate (32%). V_{max} , K_m , K_{cat} and
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26 K_{cat}/K_m values were also determined. The K_{cat}/K_m values calculated were
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28 for: farnesyl triphosphate, 166; geranyl triphosphate, 52.2; farnesyl
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30 diphosphate, 12.1; geranyl diphosphate, 8.6; isopentenyl triphosphate, 6.7;
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32 dimethylallyl diphosphate, 3.1 and isopentenyl diphosphate, 0.9. Similar
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34 results were obtained with T4 DNA ligase. The above-mentioned
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36 compounds were also substrates of firefly luciferase synthesizing the mev-
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38 pppA or mev-ppppA derivatives. In our hands, neither the acyl- or acetyl-
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40 CoA synthetases nor the ubiquitinating activating enzyme (E1) catalyzed the
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42 synthesis of ATP derivatives of these compounds. The results here
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44 presented could be related with the mechanism of action of
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46 bisphosphonates on osteoclasts or tumor cells.
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1. Introduction

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2 Bisphosphonates (P-C(R1)(R2)-P) (BPs) are widely used in the treatment of
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4 osteoporosis and other bone diseases [1]. After being fixed to bone tissues
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6 [2-4], they exert a damaging effect on osteoclasts through two classically
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8 described mechanisms: a) as substrates of different ligases generating BP
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10 derivatives of ATP through reactions 1, 5; 1a, 5a (see Table 1); this effect is
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12 supposed to be carried out by the first generation, or not containing
13
14 nitrogen, bisphosphonates (non-N-BPs) [5-9]; b) as inhibitors of the
15
16 mevalonate pathway (Fig. 1) by causing several pathological
17
18 consequences such as: inhibition of the synthesis of cholesterol and its
19
20 derivatives and, secondarily, inhibition of the post-translational modifications
21
22 (isoprenylation, farnesylation) of important regulatory proteins [10-15].
23
24 The stimulation of the synthesis of Apppl (here named iso-pppA, see
25
26 nomenclature below) in osteoclasts and J774 macrophages when treated
27
28 with nitrogen containing bisphosphonates (N-BPs) (zoledronate,
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30 risedronate, ibandronate, alendronate) has been recently reported [16].
31
32 Apppl is the compound resulting from the union of the AMP moiety of ATP
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34 with isopentenyl pyrophosphate, a component of the mevalonate pathway.
35
36 The synthesis of iso-pppA correlated well with the capacity of those BPs to
37
38 inhibit the mevalonate pathway. Moreover, iso-pppA, as well as the ATP
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40 derivative of clodronate (AppCCl₂p), inhibited the mitochondrial ADP/ATP
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42 translocase and caused apoptosis in osteoclasts [16, 17]. Apppl (iso-pppA)
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44 can then be an interesting compound to be considered in the mechanism of
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46 action of bisphosphonates. Mönkkönen et al. [16] suggested that the
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1 synthesis of iso-pppA could be catalyzed by aminoacyl tRNA synthetases
2 through reactions 1 and 6 (Table 1).
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4 The work here presented started by exploring the synthesis of iso-pppA by
5 different enzyme ligases, and has been further extended to explore the
6 synthesis of similar derivatives (mev-p_nA) of other compounds of the
7 mevalonate pathway, based on the following grounds.
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9

10 A. In our experience with enzyme ligases [18], the formation of the complex
11 E-X-AMP (or E-AMP) is rather specific for each enzyme (Table 1, reactions
12 1 or 1a). Once the E-X-AMP (or E-AMP) complex is formed, its AMP moiety
13 reacts forming a phosphodiester bond with nucleotides containing 2, 3, or
14 more terminal phosphates or even with polyphosphate chains of variable
15 length (Table 1, reactions 2, 3 and 4). However, each ligase behaves
16 differently concerning the synthesis of A(p)_nN or (p)_nA [18]; as an example,
17 the synthesis of compounds as bizarre as p₂₀A or Ap₁₆A are catalyzed by
18 luciferase in the presence of luciferin, ATP and a polyphosphate chain [19].
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21 B. Several ligases catalyze the transfer of AMP from the E-X-AMP or E-
22 AMP complexes to a bisphosphonate, with formation of the corresponding
23 derivative (reactions 5, 5a, Table 1) [8, 9].
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26 C. The interesting finding of the occurrence of Apppl (iso-pppA) in
27 osteoclasts incubated with zoledronate [16] prompted us to investigate the
28 possible synthesis by ligases of compounds of the meV-pppA type (where
29 mev represents any compound of the mevalonate pathway ending with a
30 terminal pyrophosphate, (Fig. 1)). As shown here, this synthesis can be
31 catalyzed by a variety of AMP forming ligases. This finding, apart from
32 being of potential basic interest, may help to unravel the mechanisms of
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1 action of bisphosphonates, or even may have metabolic or therapeutic
2 implications.
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7 **2. Materials and methods**

8 **2. 1. Materials**

9
10 *Mevalonate pathway compounds:* γ,γ ,dimethylallyl pyrophosphate, Cat.
11 No. D4287; farnesyl pyrophosphate, Cat. No. F6892; farnesyl triphosphate,
12 Cat. No. F1928; geranyl pyrophosphate, Cat. No. G6772; geranyl
13 triphosphate, Cat. No. G2418; isopentenyl pyrophosphate, Cat. No. I0503,
14 and isopentenyl triphosphate, Cat. No. I1282, were from Sigma.
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24 *Enzyme ligases:* T4 RNA ligase (EC 6.5.1.3) was from BioLabs
25 (M0204S, lots 40 and 0410802), with a specific activity of 12,000 U/mg [8].
26
27 T4 DNA ligase (EC 6.5.1.1) from *E. coli*, was from Roche (Cat No.
28 10716359001, lot 12407122) with a specific activity of 3,000 U/mg [9]; acyl-
29 CoA synthetase (EC 6.2.1.3), Ref. A-3352 (lot 115K11611); acetyl-CoA
30 synthetase (EC 6.2.1.1), Ref. A-1765 (lot 045K1431); Ub activating
31 enzyme (E1) Ref. U1758 (lot 027K1384) and luciferase (EC 1.13.12.7),
32 Ref. L-9506 (lot 66H8275) were from Sigma.
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44 *Other enzymes:* Yeast inorganic pyrophosphatase (EC 3.6.1.1) was
45 from BioLabs (Cat. No. 2403); shrimp alkaline phosphatase (EC 3.1.3.1)
46 was from Roche (Ref. 1758250); phosphodiesterase I from *crotalus*
47 *durissus* was from Boehringer Mannheim, Germany (now Roche).
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54 *Other materials:* Sodium tripolyphosphate (P₃) was from Sigma (Ref. T-
55 5633). [³²P]ATP 3000 Ci/mmol) was from Perkin Elmer. TLC silica-gel
56 fluorescent plates were from Merck. Radioactively labelled nucleotides
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1 were quantified with the help of a Typhoon Trio from GE Healthcare. HPLC
2 was carried out in a Hewlett Packard chromatograph (model 1090) with a
3 diode array detector. The Hypersil ODS column (4.6 x 100 mm) was from
4 Hewlett Packard.
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10 11 *2.2 Synthesis of ATP derivatives of compounds of the mevalonate* 12 *pathway catalyzed by ligases* 13

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15 *T4 RNA ligase.* The reaction mixtures (0.02 ml) contained 50 mM
16 Tris/HCl (pH 7.4), 1 mM dithiothreitol (DTT), 5 mM MgCl₂, 0.1 U of
17 pyrophosphatase, 0.02 mM [α -³²P]ATP (0.4 μ Ci), 1 mM
18 phosphoenolpyruvate, 0.2 U pyruvate kinase, compounds of the
19 mevalonate pathway and enzyme as indicated. After incubation at 30°C the
20 reaction mixtures were analysed by thin-layer chromatography (TLC) or
21 high-pressure liquid chromatography (HPLC) [8].
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36 *T4 DNA ligase.* The reaction mixtures (0.02 ml) contained 50 mM
37 HEPES/KOH (pH 7.2), 1 mM DTT, 5 mM MgCl₂, 0.1 U of pyrophosphatase,
38 0.02 mM [α -³²P]ATP (0.4 μ Ci), compounds of the mevalonate pathway and
39 enzyme as indicated. After incubation at 30°C the reaction mixtures were
40 analysed by TLC [9].
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49 *Luciferase.* The reaction mixtures (0.02 ml) contained 50 mM
50 HEPES/KOH (pH 7.5), 5 mM MgCl₂, 0.1 U of pyrophosphatase, 0.02 mM [α -
51 ³²P]ATP (0.4 μ Ci), 0.02 mM D-luciferin, compounds of the mevalonate
52 pathway and enzyme as indicated. The enzyme was diluted in 0.5 M
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1 Hepes/KOH (pH 7.5)/10% glycerol/1 mM DTT. After incubation at 30°C the
2 reaction mixtures were analysed by TLC.
3

4 *Other enzymes.* acyl-CoA synthetase [20], acetyl-CoA synthetase [21]
5 and the ubiquitin activating enzyme (E1) [22] were assayed as previously
6 described.
7

8 *Enzyme units (U).* One unit (U) is the amount of enzyme transforming
9 one μmol of substrate per min.
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11 *Analysis by TLC.* Aliquots of 1.5 μl of the reaction mixtures were spotted
12 on silica gel plates and developed for 2 h in dioxane:ammonium
13 hydroxide:water (6:1:6 by volume).
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17 2.3 Nomenclature.

18 The compounds here synthesized are structurally related to dinucleoside
19 polyphosphates ($\text{Np}_n\text{N}'$) or nucleoside(5')oligophospho(5')nucleoside or
20 $\text{N}(5')\text{p}_n(5')\text{N}'$. There are a plethora of such compounds, some of them of
21 physiological relevance [23-25]. Most of their abbreviations start and end
22 with a capital letter representing a nitrogenous base, for instance Ap_4A or
23 AppppA or diadenosine tetraphosphate. For a review on the chemistry and
24 nomenclature of dinucleoside polyphosphates see [26].
25

26 The recently discovered ATP derivative of isopentenyl diphosphate has
27 been abbreviated as Apppl (I standing for isopentenyl) and named as 1-
28 adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester [16]. In this report, to avoid
29 confusion with the already well-established nomenclature for the
30 dinucleoside polyphosphates, the ATP derivatives of the compounds of the
31 mevalonate pathway have been abbreviated using the first three letters of
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its name, with a dash followed by the number of inner phosphates and the suffix adenosine. So, the ATP derivatives of isopentenyl diphosphate or of farnesyl triphosphate have been abbreviated as iso-pppA or far-ppppA, respectively. In general, the compounds of the mevalonate pathway covalently bound to AMP are named as mev-(p)_nA. (Table 1).

3. Results

3.1 Synthesis and characterization of isopentenyl triphospho adenosine (iso-pppA) by T4 RNA ligase. Analysis by TLC.

To investigate whether the ligases capable to synthesize bisphosphonate derivatives of ATP, as already shown in our laboratory [8, 9], were able to synthesize as well, the ATP derivative of isopentenyl pyrophosphate (Apppl [16], here iso-pppA), the enzyme T4 RNA ligase was firstly tested. The reaction mixture contained of 0.02 mM [α -³²P]ATP and 1 mM iso-PP.(Fig. 2, lanes 3,5,and 7). At different times of incubation samples were taken and analyzed by TLC as described in Materials and methods. Two control reactions (with no added enzyme (Fig. 2, lane 1) and with no added iso-PP (Fig. 2, lanes 2, 4 and 6), were run in parallel. In the reaction mixture containing iso-PP (Fig. 2, lanes 3, 5 and 7) a new radioactive spot migrating ahead of AMP and increasing with incubation time was formed. This spot, the presumptive iso-pppA derivative, was not degraded by alkaline phosphatase (lane 9) and, after inactivation of the phosphatase, treatment with snake venom phosphodiesterase (SVP) rendered labeled AMP. In our

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view, these results indicate that the synthesis of iso-pppA catalyzed by T4 RNA ligase takes place through reactions 1a and 6a (Table 1).

In the control reactions (lanes 2, 4 and 6), containing T4 RNA ligase but not iso-PP, the formation of radioactive spots that increased with incubation time and co-migrated with standards of AMP and Ap₄A, were also observed. Treatment with alkaline phosphatase, resulted in the appearance of a new spot (that eluted slightly above a standard of ADP) and was resistant to phosphodiesterase. This product, unrelated to the presence or absence of isopentenyl-PP, could not be identified.

3.2. Synthesis and characterization of isopentenyl tetraphospho adenosine (iso-ppppA) by T4 RNA ligase. Analysis by HPLC

Reaction mixtures (0.08 ml) containing 60 U T4 RNA ligase, 0.3 mM ATP and 0.4 mM Iso-PPP (other components as indicated in Material and methods), were incubated for 6 and 20 h, and aliquots analyzed by HPLC (Fig. 3, b and c). The rest of the reaction mixture was treated with 1 μ l (1U) of shrimp alkaline phosphatase (AP) (for 2 h at 37°C) (Fig. 3, d) and, after inactivation of the phosphatase by heating, treated with snake venom phosphodiesterase (SVP) (Fig. 3, e). The control reaction with no added enzyme is shown in (Fig. 3, a). As shown here, the presumptive iso-ppppA, (with a retention time (t_R) of around 54 min), was resistant to the phosphatase and yielded AMP when incubated with phosphodiesterase (Fig. 3, e). In the course of the reaction, the synthesis of a compound (t_R of 34 min) that co-migrated with a standard of Ap₄A (not shown) was also observed (Figs. 3, b, c and d).

3.3. A comparative study of several compounds of the mevalonate pathway
as substrates of T4 RNA ligase

On the accounts presented in the Introduction, the following compounds of the mevalonate pathway were assayed comparatively as substrates of T4 RNA ligase, at two different concentrations (0.3 and 0.8 mM): isopentenyl triphosphate (iso-PPP); isopentenyl diphosphate (iso-PP); farnesyl triphosphate (far-PPP); farnesyl diphosphate (far-PP); geranyl triphosphate (ger-PPP); geranyl diphosphate (ger-PP) and dimethylallyl diphosphate (dim-PP). As shown in Fig.4, new radioactive spots corresponding to the ATP derivatives of the above substrates were clearly observed. The initial velocity values, expressed as mU/mg of protein (mean of 2-4 determinations) were calculated and represented in Fig. 5. The relative activities (%) (calculated at the 0.3 mM concentration) to synthesize the corresponding meV-p_nA derivatives of the following substrates were: ger-PPP, 100 % or 7.6 mU/mg; iso-PPP, 42 %; far-PPP, 39%; ger-PP, 24%; far-PP, 18 %; dim-PP, 12 % and iso-PP, 6 %. Inhibition by excess of substrate was observed with far-PP, far-PPP and ger-PPP; the enzyme activities determined with these substrates at 0.8 mM were <1%, 7% and 68%, respectively, of those obtained at 0.3 mM (Figs. 4 and 5). The inhibition was also followed at different concentrations of substrate (far-PP and far-PPP) as shown in Fig. 6a,b, and Fig.6c,d, respectively.

The V_{max} , K_m , K_{cat} and K_{cat}/K_m values of T4 RNA ligase determined for each one the substrates tested are compiled in Table 2. The V_{max} values were higher for the substrates with a terminal triphosphate. Regarding K_m values, iso-PP and iso-PPP were the substrates with the lowest affinity for the

1 enzyme (K_m values around 4 mM), while the higher efficacy (K_{cat}/K_m) was
2 found for far-PPP, a compound whose derivative formation is inhibited by
3
4 an excess of substrate (see Figs. 4, 5 and 6c, d).
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8 *3.4 Synthesis of ATP derivatives of compounds of the mevalonate pathway* 9 10 *catalyzed by T4 DNA ligase*

11 This point started with the synthesis and characterization of iso-pppA using
12 iso-PP as substrate. The reaction mixtures contained 0.02 mM [α - 32 P]ATP,
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14 1 mM iso-PP, and other components as indicated in Material and methods
15
16 (Fig. 7). Samples were taken from the reaction mixture at different times of
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18 incubation and analyzed by TLC. As performed with T4 RNA ligase (Fig. 2),
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20 two control reactions (with no added enzyme (Fig. 7, lane 1) and with no
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22 added iso-PP (Fig. 7, lanes 2, 4 and 6), were run in parallel. In the reaction
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24 mixture containing iso-PP (Fig. 7, lanes 3, 5 and 7) a new spot migrating
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26 ahead of AMP and increasing with incubation time was formed. This spot
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28 (presumptive iso-pppA), as with T4 RNA ligase (Fig. 2), was not degraded
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30 by alkaline phosphatase (AP) (lane 9), and treatment with snake venom
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32 phosphodiesterase (SVP) rendered AMP, demonstrating the synthesis of
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34 iso-pppA from ATP and iso-PP. As occurred with T4 RNA ligase (Fig. 2),
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36 the control reactions with added enzyme but not iso-PP (Fig. 7, lanes 2, 4
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38 and 6), showed also spots that co-migrated with standards of AMP and
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40 Ap_4A . After alkaline phosphatase treatment, a spot resistant to
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42 phosphodiesterase, eluting slightly above a standard of ADP, was also
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44 observed.
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57 Before comparing the results obtained with T4 RNA ligase (Fig. 2) and T4
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59 DNA ligase (Fig. 7), it should be remind that the T4 RNA ligase assays
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include an ATP regenerating system, due to the ATPase activity present in the commercial preparation of T4 RNA ligase [8]. Therefore, ADP (an usual contaminant of the [α - 32 P]ATP preparation) is not observed, while it is clearly appreciated in the T4 DNA ligase assays that do not include the ATP regenerating system. Since both enzymes are able to catalyze the synthesis of Ap₄A from ATP [27, 28], a small amount of Ap₄A used to be formed in the absence or presence of iso-PP. However, in the context of this work the important points are: i) the appearance of a product, present exclusively in the reaction mixtures containing iso-PP; ii) its resistant to alkaline phosphatase and iii) the formation of AMP following treatment with phosphodiesterase.

All the compounds tested as substrates of T4 RNA ligase (see above) were also assayed as substrates of T4 DNA ligase, at a concentration of 0.3 and 0.8 mM, and the initial velocities determined (Fig. 8). At 0.3 mM the relative velocities (%) to synthesize the corresponding meV-p_nA derivatives were for: far-PPP, 100 % or 25.1±0.9 mU/mg); iso-PPP, 89%; ger-PPP, 78 %; iso-PP, 17%); dim-PP, 14 %; far-PP, 7 % and ger-PP, 2%. Inhibition (%) of the activity by 0.8 mM substrate vs. 0.3 mM was also observed with far-PPP (81%) and far-PP (50%) (Fig. 8).

For the V_{max} , K_m , K_{cat} and K_{cat}/K_m values calculated for T4 DNA ligase and the mevalonate compounds see Table 3. Again, the maximum catalytic efficiency (K_{cat}/K_m) was obtained for far-PPP as a substrate. In general, the efficacy of T4 DNA ligase to synthesize meV-p_nA derivatives is better than with T4 RNA ligase (compare the K_{cat}/K_m values for both enzymes in Tables 2 and 3).

3.4 Synthesis of mevalonate derivatives of ATP by other ligases

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2 Luciferase, acetyl- and acyl-CoA synthetases, and the ubiquitin activating
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4 enzyme (E1), previously assayed in the synthesis of both (di)nucleoside
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6 polyphosphates and ATP derivatives of bisphosphonates [18, 22], were also
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8 tested for the synthesis of mevalonate derivatives. From these enzymes,
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10 luciferase was the only one able to catalyze the synthesis of these
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12 derivatives. As previously known, luciferase (E), in the presence of D-
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14 luciferin and ATP, forms the complex E-luciferin-AMP, the AMP moiety may
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16 be donated to nucleosides containing at least a triphosphate chain, and
17
18 the synthesis of dinucleoside polyphosphates occurs (Table 1, reactions 1,
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20 2) [18]. However when the ATP concentration in the assay is very low (μM),
21
22 the enzyme behaves as an ATPase liberating AMP [29, 30]. The synthesis
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24 of ATP mevalonate derivatives was approached here, incubating luciferase
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26 in the presence of 0.02 mM [α - ^{32}P]ATP and iso-PPP, far-PPP or ger-PPP at
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28 0.3 mM each (Fig. 9, A); a control in the absence of added substrate was
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30 run in parallel. After 20 min incubation, the reaction mixtures were treated
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32 with alkaline phosphatase. In addition to an important amount of AMP and a
33
34 minor amount of Ap_4A , the synthesis of the corresponding ATP derivatives
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36 was also observed. Formation of adenosine 5'-tetraphosphate (p_4A) was
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38 detected with far-PPP as substrate, revealing the occurrence of free
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40 triphosphate in this commercial preparation. The radioactive spots
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42 corresponding to the presumptive mevalonate-ppppA were not sensitive to alkaline
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44 phosphatase as shown in Fig. 9, B and sensitive to phosphodiesterase,
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46 yielding AMP as one of the products (results not shown). A similar
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48 experiment using iso-PP as substrate is shown Fig. 9, C. The iso-pppA
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1 synthesized was not affected by treatment with alkaline phosphatase. The
2 initial velocities calculated for the synthesis of the ATP derivatives shown in
3 Fig. 9 were for: iso-PPP (2.0 mU/mg); far-PPP (1.8 mU/mg); ger-PPP (3.1
4 mU/mg) and iso-PP (0.16 mU/mg). Luciferase prefers the triphosphate
5 derivatives of the compounds of mevalonate pathway, equally as it prefers
6 nucleosides with a terminal P₃ over those with a terminal P₂, as substrates
7 for the synthesis of dinucleoside polyphosphates [18].
8

9 In our hands, the other ligases tested, i.e. acetyl-CoA synthetase, acyl-CoA
10 synthetase, and the ubiquitin activating enzyme E1 did not catalyze the
11 synthesis of ATP derivatives with any one of the mevalonate compounds
12 here mentioned.
13

14 **4. Discussion**

15 Our previous work on the synthesis of dinucleoside polyphosphates [18]
16 and on ATP derivatives of bisphosphonates catalyzed by ligases [8, 9], and
17 the finding by Mönkkönen of the occurrence of isopentenyl-
18 triphosphoadenosine (Apppl, here iso-pppA) in osteoclasts cells treated
19 with bisphosphonates [16], prompted us to study the synthesis of ATP
20 derivatives (mev-pppA and mev-ppppA) by several ligases. About the
21 potential relationship between the effect of bisphosphonates both on the
22 synthesis of these compounds in particular and on osteoclasts in general,
23 two main possibilities can be envisaged.
24

25 A. Bisphosphonates could stimulate the synthesis of mev-pppA considering
26 simply that, if BPs accumulate in bone tissues inhibiting some of the
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1 enzymes of the mevalonate pathway (Fig. 1), an increase in the
2 concentration of metabolites upstream the inhibited step would take place,
3
4 and consequently, an increase of the synthesis of the mevalonate
5 derivatives (mev-pppA) catalyzed by ligases, could occur (Table 1).
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9 Recently, several laboratories have studied the interaction of alendronate,
10 ibandronate, risedronate and zoledronate (bisphosphonates of the second
11 and third generation, all N-BPs), with crystallized human farnesyl
12 pyrophosphate synthetase (Fig. 1) with similar and complementary results
13 [31-33]. The nitrogen atom of N-BPs interacts with the Thr-201 and Lys-200
14 of the enzyme, located in its carbocation-binding site favoring the position of
15 BPs in the dimethylallyl/geranyl diphosphate ligand pocket [32]. Those N-
16 BPs bind to this site through a trinuclear metal center similar to that formed
17 with the terminal pyrophosphate of some compounds of the mevalonate
18 pathway [33]. In addition, the isopentenyl diphosphate accumulated (due to
19 the inhibition of the enzyme), rather than competing for and displacing the
20 BP, binds and stabilizes the enzyme-N-BP complex, forming a ternary
21 complex. This fact contributes to the exceptional *in vivo* efficacy of the N-BP
22 drugs [33], and could explain the preferential synthesis of iso-pppA reported
23 by Mönkkönen [16]. At any rate, both the N-atom and the bisphosphonate
24 chain itself are critical for the inhibitory effect of N-BP on this enzyme. In
25 this context, we may recall that *the components of the mevalonate pathway*
26 *comprise the highest gathering of consecutive compounds with a terminal*
27 *pyrophosphate in any known metabolic pathway in mammals*, what could
28 be another characteristics for the preferential action of bisphosphonates in
29 this pathway (Fig. 1). It may also be recalled that some BPs are substrates
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for the synthesis of ATP derivatives of the type AppC(R1)(R2)p catalyzed by ligases [5-9]; in principle, these ATP derivatives could interfere with the activity of enzymes using ATP as substrate. To this group belong at least 150 enzymes with transferase activity and more than 90 with ligase activity (see Enzyme Nomenclature). Probably most of these ATP derivatives could act on these enzymes as competitive inhibitors (although other type of inhibition could also be contemplated) with variable efficacy. In this regard it is worthy noting that, *the mevalonate pathway presents the exceptional characteristic of holding three enzymes (2 kinases and 1 decarboxilase) requiring ATP and operating in sequence* (Fig. 1). The concatenation of these steps could make the mevalonate pathway specially exposed to the effect of the BP derivatives of ATP.

B. The synthesis of mev-pppA is partially or totally unrelated to the effect of bisphosphonates. The occurrence of mev-pppA in liver and other tissues cannot be disregarded in view of the quantity of ligases potentially able to transfer the AMP moiety of the E-AMP or EX-AMP complexes to compounds with a terminal di or triphosphate [18]. Examples of this possibility are here reported for the case of T4 RNA ligase, T4 DNA ligase and luciferase. Moreover, the synthesis of compounds with inner phosphate(s) and containing at least a terminal nucleotide (AMP, GMP, UMP or CMP, depending on the ligase involved) can be equally pondered. These compounds can be considered as metabolic by-products, with no metabolic significance, whose natural destiny is to be eliminated by specific or unspecific enzymes and, because of the low concentration reached in the cell, have passed unnoticed. Experiments performed years ago in our

laboratory showed the existence of a number of still unknown nucleotides with these peculiarities in rat liver [34]. However, in our view, compounds present at low concentration in tissues and containing inner phosphate(s) are also candidates to be regulatory molecules [35]. In the particular case of the mevalonate derivative, iso-pppA, this compound inhibited the ADP/ATP translocase and caused apoptosis in osteoclasts [16]. The possibility that other mev-pppA derivatives could also have regulatory effects remains an open question.

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10 LEGEND TO FIGURES

11 Fig. 1. Components of the mevalonate pathway, its precursors and some
12 metabolic connections. The metabolic pathway from acetyl-CoA to
13 mevalonate-5-phosphate, and the implicated enzymes (1-5) are indicated in
14 the figure. The enzymes are: 1, acetyl-CoA acetyltransferase (EC 2.3.1.9);
15 2, hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5); 3, 3-
16 hydromethylglutaryl-CoA reductase, (EC 1.1.1.34); 4, mevalonate kinase
17 (EC 2.7.1.36); 5, phosphomevalonate kinase (EC 2.7.4.2). Note that the
18 three consecutive steps catalyzed by enzymes (4), (5) and (A), consume
19 three moles of ATP. The inset in the figure comprises the compounds of the
20 mevalonate pathway and the enzymes (A-G) involved: A,
21 diphosphomevalonate decarboxylase, (EC 4.1.1.33); B, isopentenyl-
22 diphosphate D-isomerase, (EC 5.3.3.2); C, dimethylallyltransferase,
23 (EC 2.5.1.1); D, geranyltransferase (farnesyl-diphosphate synthase),
24 (EC 2.5.1.10); E, farnesyltransferase, (EC 2.5.1.29); F, farnesyl-
25 diphosphate farnesyltransferase, (EC 2.5.1.21). Note as the two activities
26 catalyzed by the human farnesyl pyrophosphate synthetase (see the text,
27 [31-33]) are registered in the Enzyme Commission as carried out by the
28 enzymes with entries EC 2.5.1.1 and EC 2.5.1.10. To emphasize the
29 occurrence of pyrophosphate in all the compounds of the cycle, this moiety

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1 has been marked out (•). Some of the great variety of compounds

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5 synthesized from those metabolites, and essential for cell function, are
6 encircled by a dotted line.
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11 Fig. 2. Synthesis of isopentenyl-triphospho(5')adenosine (iso-pppA) from
12 isopentenyl diphosphate and ATP, catalyzed by T4 RNA ligase. The
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14 reaction was carried out in the presence of 0.02 mM [$\alpha^{32}\text{P}$]ATP (0.4 μCi), 1
15 mM iso-PP, 20 U enzyme and other components as specified in Materials
16 and methods (lanes 3, 5 and 7). Two control reactions (with no added
17 enzyme (lane 1) and with no added isopentenyl-PP (lanes 2, 4 and 6) were
18 run in parallel. Samples were taken after 15, 30 and 60 min incubation and
19 subjected to TLC. After 60 min incubation, the reaction mixtures containing
20 enzyme (lanes 6 and 7) were treated with 1 μl (1U) of shrimp alkaline
21 phosphatase (AP) (lanes 8 and 9). Once the phosphatase was denatured
22 by heating at 90°C for 6 min, the reaction mixture was treated with snake
23 venom phosphodiesterase (SVP) (lanes 10 and 11).
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44 Fig. 3. Synthesis and characterization of isopentenyl-
45 tetraphospho(5')adenosine (iso-ppppA) from isopentenyl triphosphate and
46 ATP, catalyzed by T4 RNA ligase. Analysis by HPLC.
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50 Reaction mixtures (0.08 ml) containing 60 U T4 RNA ligase, 0.3 mM ATP
51 and 0.4 mM Iso-PPP (other components as indicated in Material and
52 methods) were incubated for 6 h (b) and 20 h (c) and aliquots analyzed by
53 HPLC. The rest of the reaction mixture was treated with 1 μl (1U) of shrimp
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1 alkaline phosphatase (for 2h at 37°C) (d) and, after inactivation of the
2 phosphatase by heating, further treated with snake venom
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4 phosphodiesterase (e). A control reaction with no added enzyme is shown
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7 in (a).
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11 Fig. 4. Synthesis of ATP derivatives of compounds of the mevalonate
12 pathway (mevp_nA), catalyzed by T4 RNA ligase. The indicated compounds
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14 were tested as substrates of T4 RNA ligase at two different concentrations:
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16 0.3 mM (upper panel) and 0.8 mM (lower panel), in the presence of 0.02
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18 mM [α^{32} P]ATP (0.4 μ Ci) and 16 U enzyme. Controls with no added
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20 substrate were run in parallel (first lanes).
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29 Fig. 5. Synthesis of the ATP derivatives of compounds of the mevalonate
30 pathway (mevp_nA), catalyzed by T4 RNA ligase. Relative activities. The
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32 substrates were assayed at two different concentrations: 0.3 mM or 0.8 mM,
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34 as described in Fig. 4. The ordinate scale reflects enzyme activity (initial
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36 velocity) calculated as mU/mg of protein.
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44 Fig. 6. Inhibition by excess of substrate, of the synthesis of far-pppA and
45 far-ppppA, catalyzed by T4 RNA ligase, The reaction mixtures (0.02 ml)
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47 contained 0.02 mM [α^{32} P]ATP (0.4 μ Ci), far-PP and 20 U enzyme (A), or
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49 far-PPP and 13 U enzyme (C). Other components and analysis by TLC as
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51 indicated in Materials and methods. The corresponding derivatives formed
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57 in the assay (pmoles), were calculated and are represented in (B) and (D).
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Fig. 7. Synthesis of isopentenyl-triphospho(5')adenosine (iso-pppA) catalyzed by T4DNA ligase. The reaction was carried out in the presence of 0.02 mM [$\alpha^{32}\text{P}$]ATP (0.4 μCi), 1 mM iso-PP, 2 U enzyme, and other components as specified in Materials and methods (lanes 3, 5, 7). Two control reactions (with no added enzyme (lane 1) and with no added iso-PP (lanes 2, 4 and 6) were run in parallel. Samples were taken after 15, 30 and 60 min incubation and subjected to TLC. After 60 min incubation, the reaction mixtures containing enzyme (lanes 6 and 7) were treated first with alkaline phosphatase (AP) (lanes 8 and 9) and, after inactivation of the phosphatase, treated with snake venom phosphodiesterase (SVP) (lanes 10 and 11).

Fig. 8. Synthesis of the ATP derivatives of compounds of the mevalonate pathway (mevp_nA) catalyzed by T4 DNA ligase. Relative activities. The substrates were assayed in the presence of 0.02 mM [$\alpha^{32}\text{P}$]ATP (0.4 μCi) at two different concentrations: 0.3 mM or 0.8 mM, and 2 U enzyme. The ordinate scale reflects enzyme activity (initial velocity) calculated as mU/mg of protein.

Fig. 9. Synthesis of ATP derivatives of the mevalonate pathway compounds, catalyzed by firefly luciferase. The reaction mixtures contained 0.02 mM [$\alpha^{32}\text{P}$]ATP (0.4 μCi), and 0.3 mM iso-PPP, far-PPP or ger-PPP (A), or 0.4 mM iso-PP (C), and 5 μg enzyme. Controls in the absence of mevalonate compounds were carried out (lanes 1). Samples were taken after 20

1 min incubation and subjected to TLC (A and C). The reaction mixtures were
2 further treated with alkaline phosphatase (see B and D).
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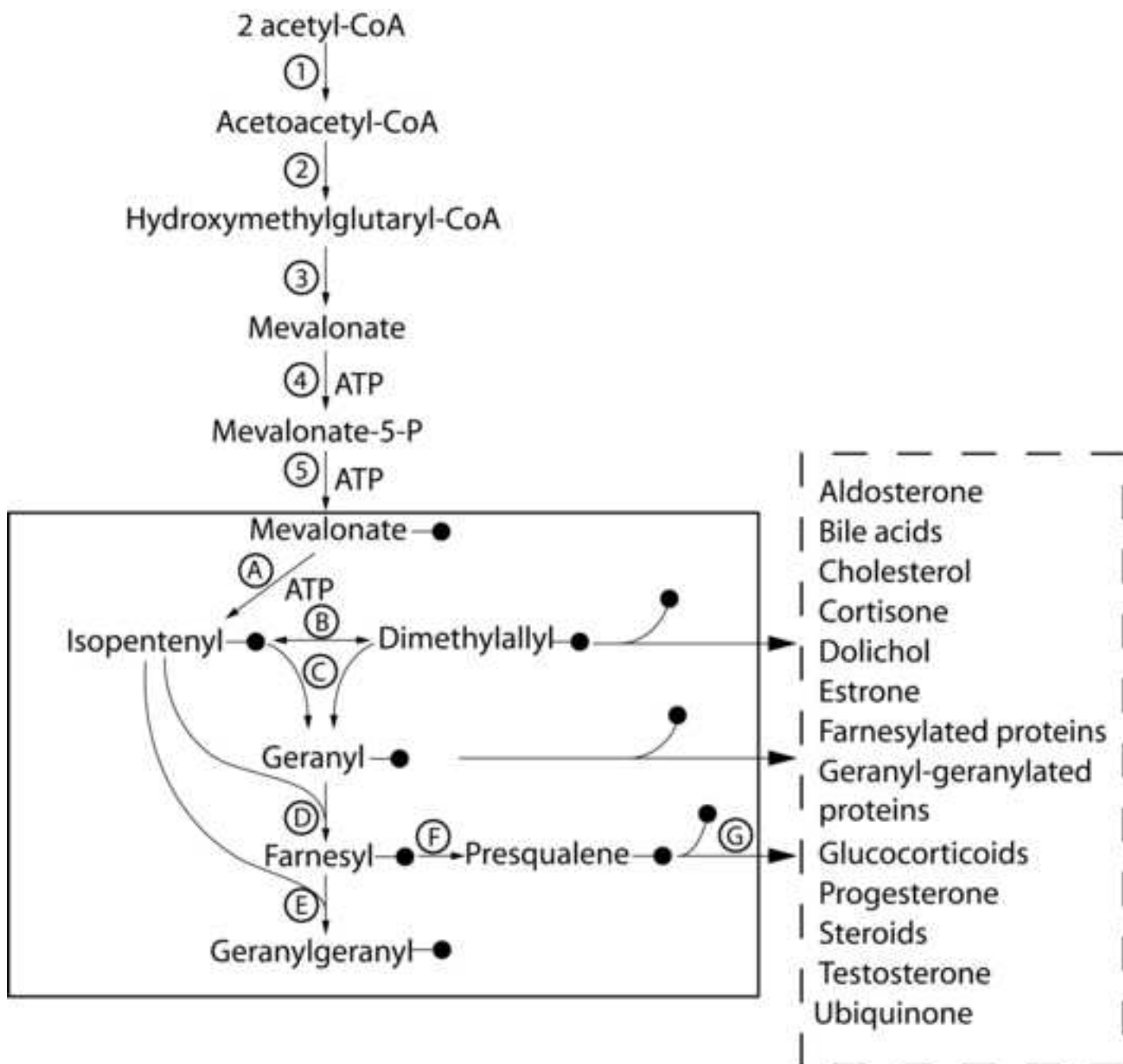


Fig. 1

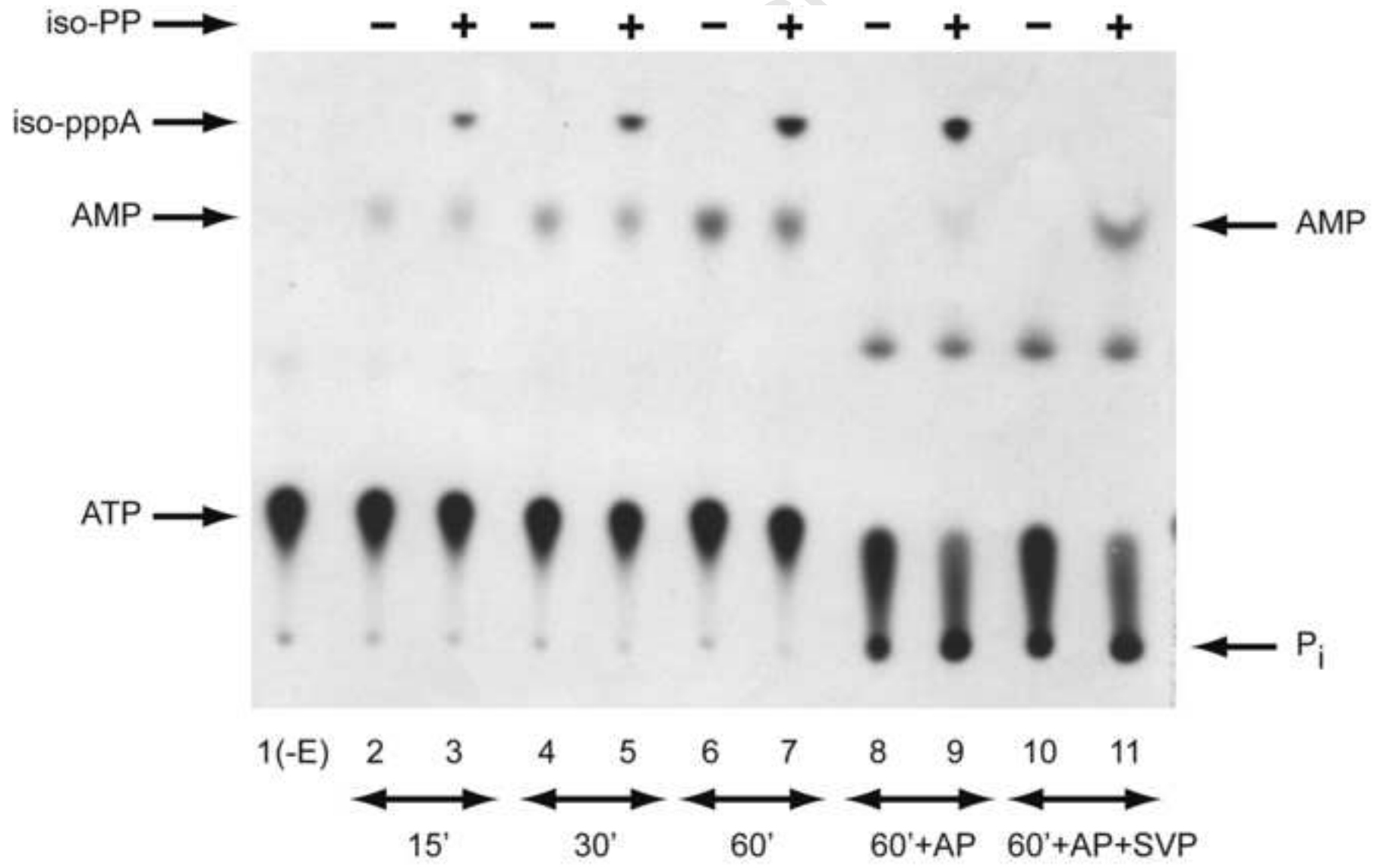


Fig. 2

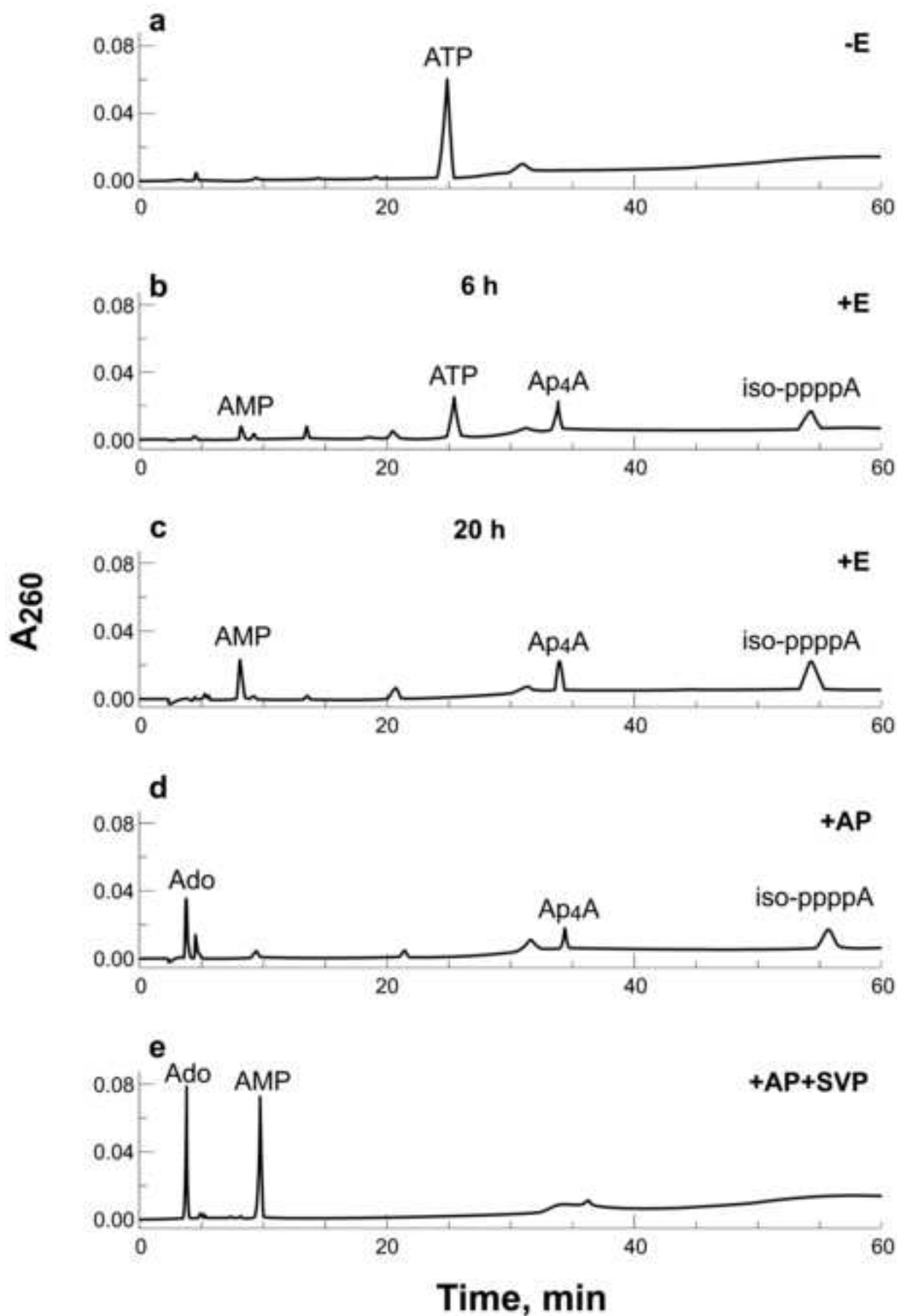


Fig3

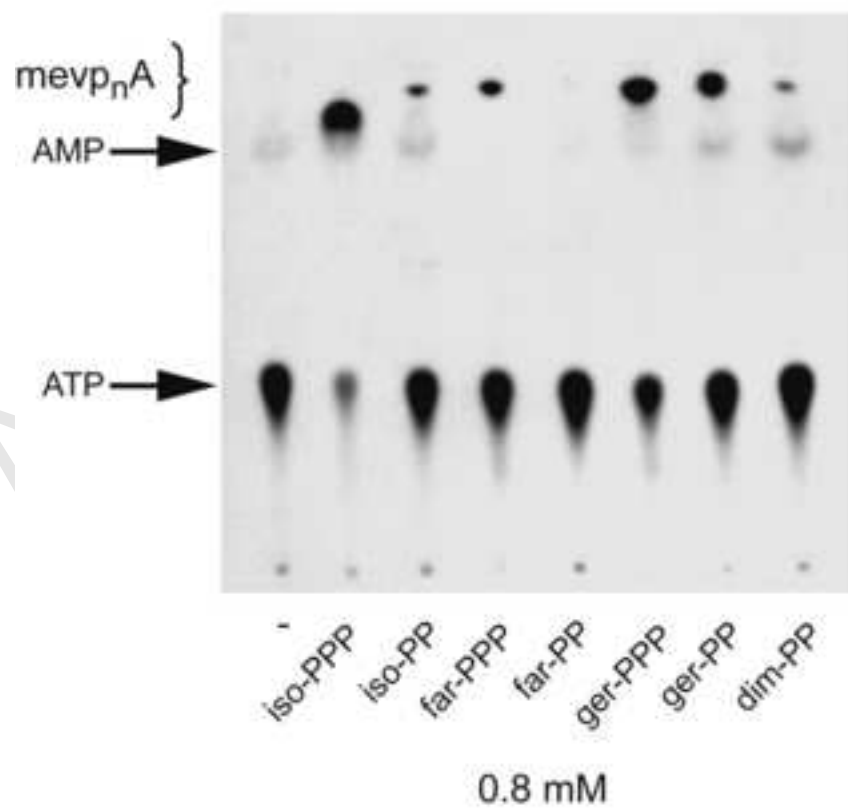
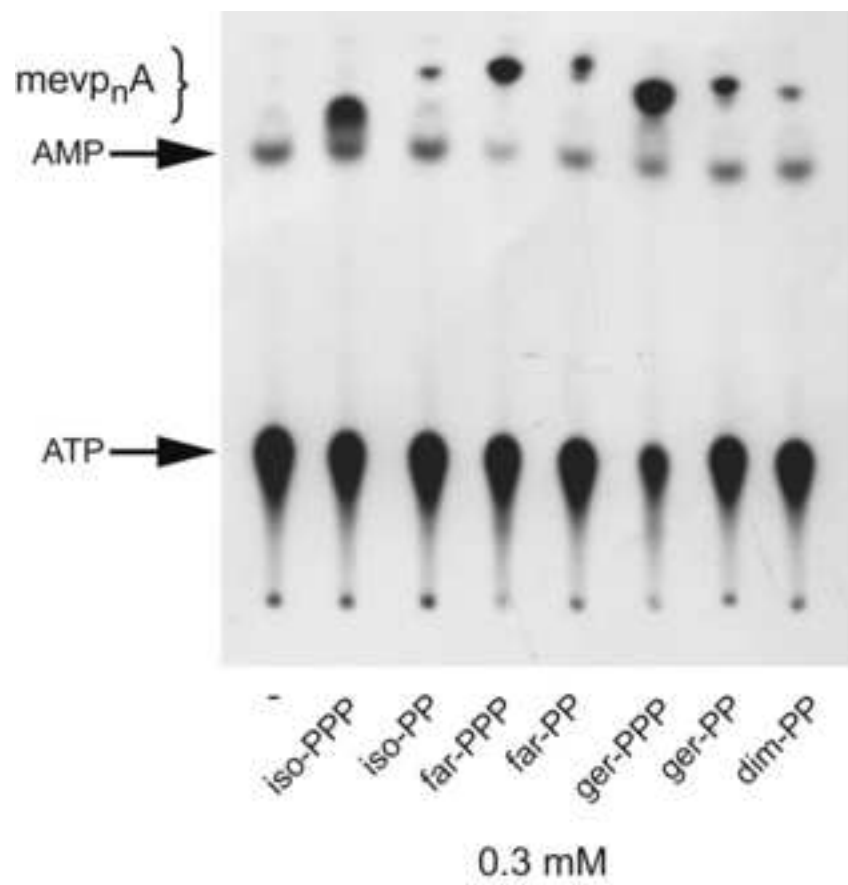


Fig.4

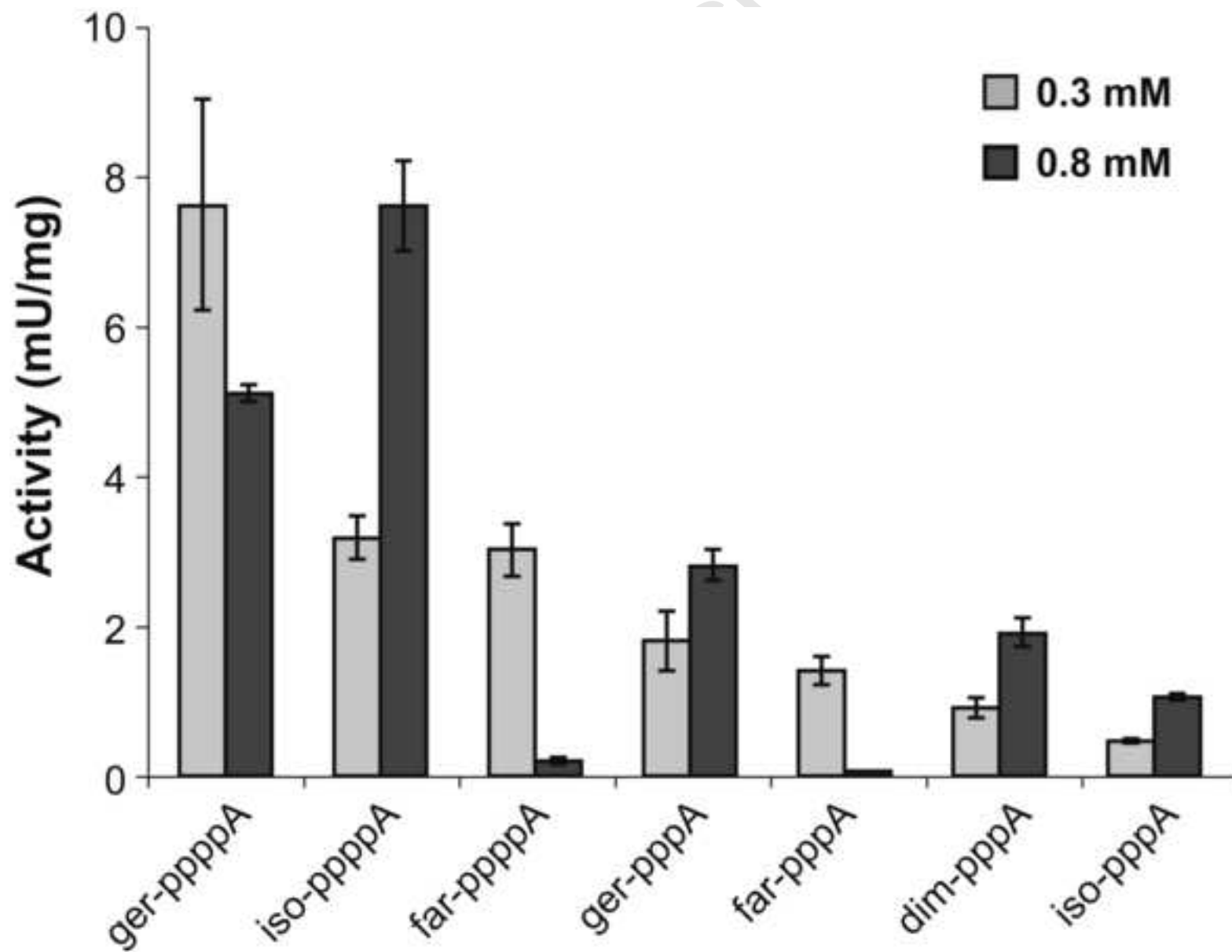


Figure 5

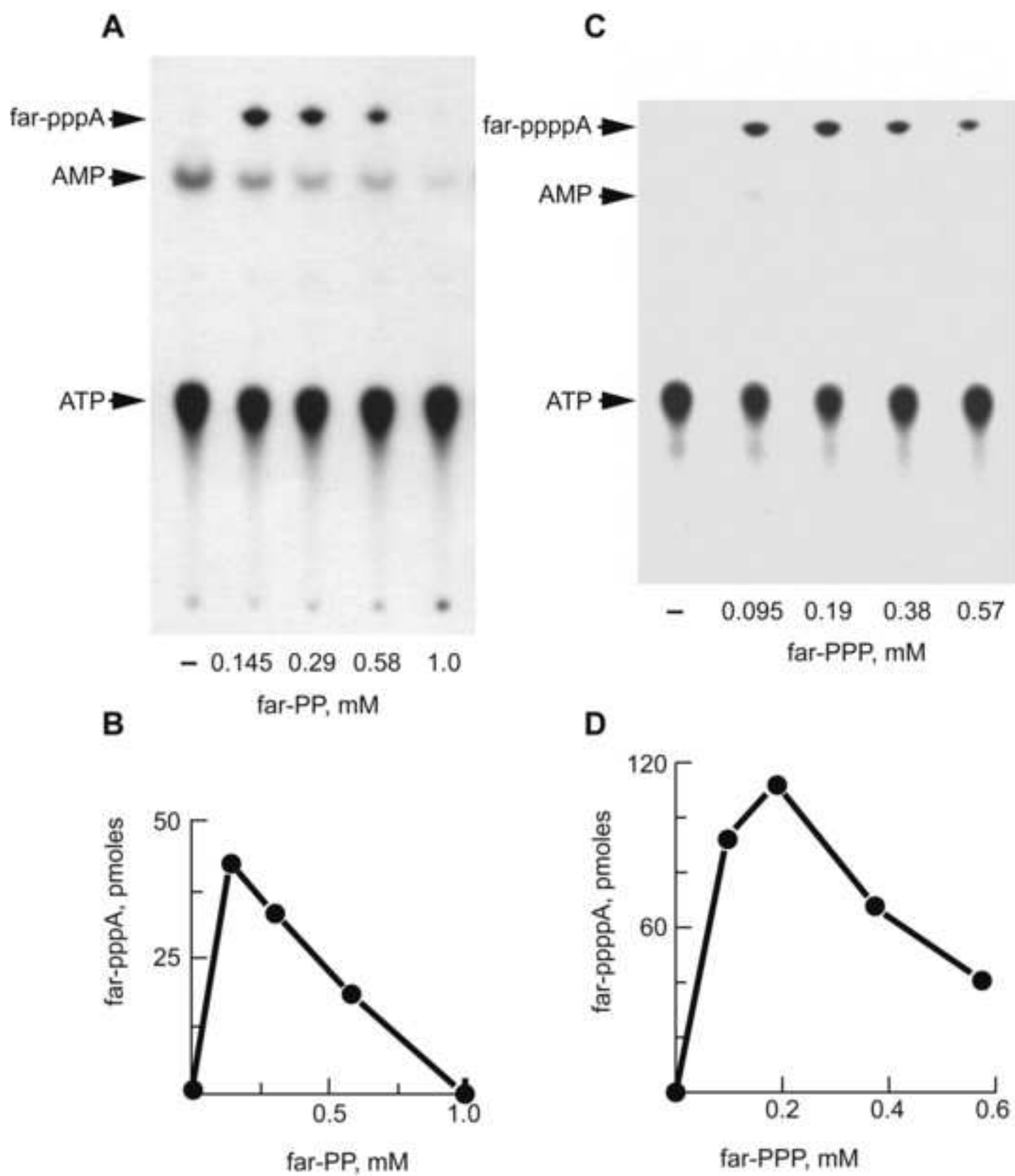


Fig.6

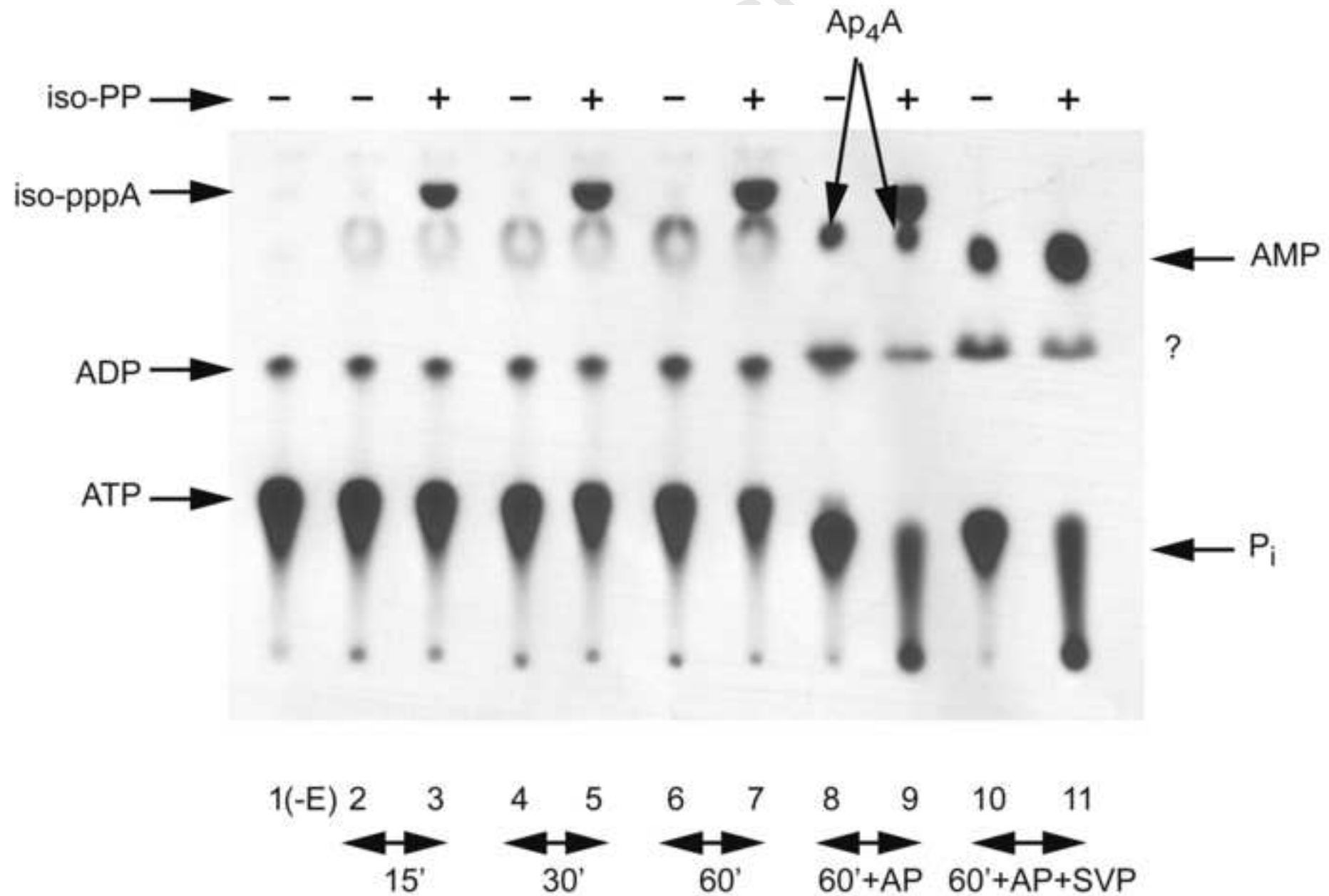


Fig. 7

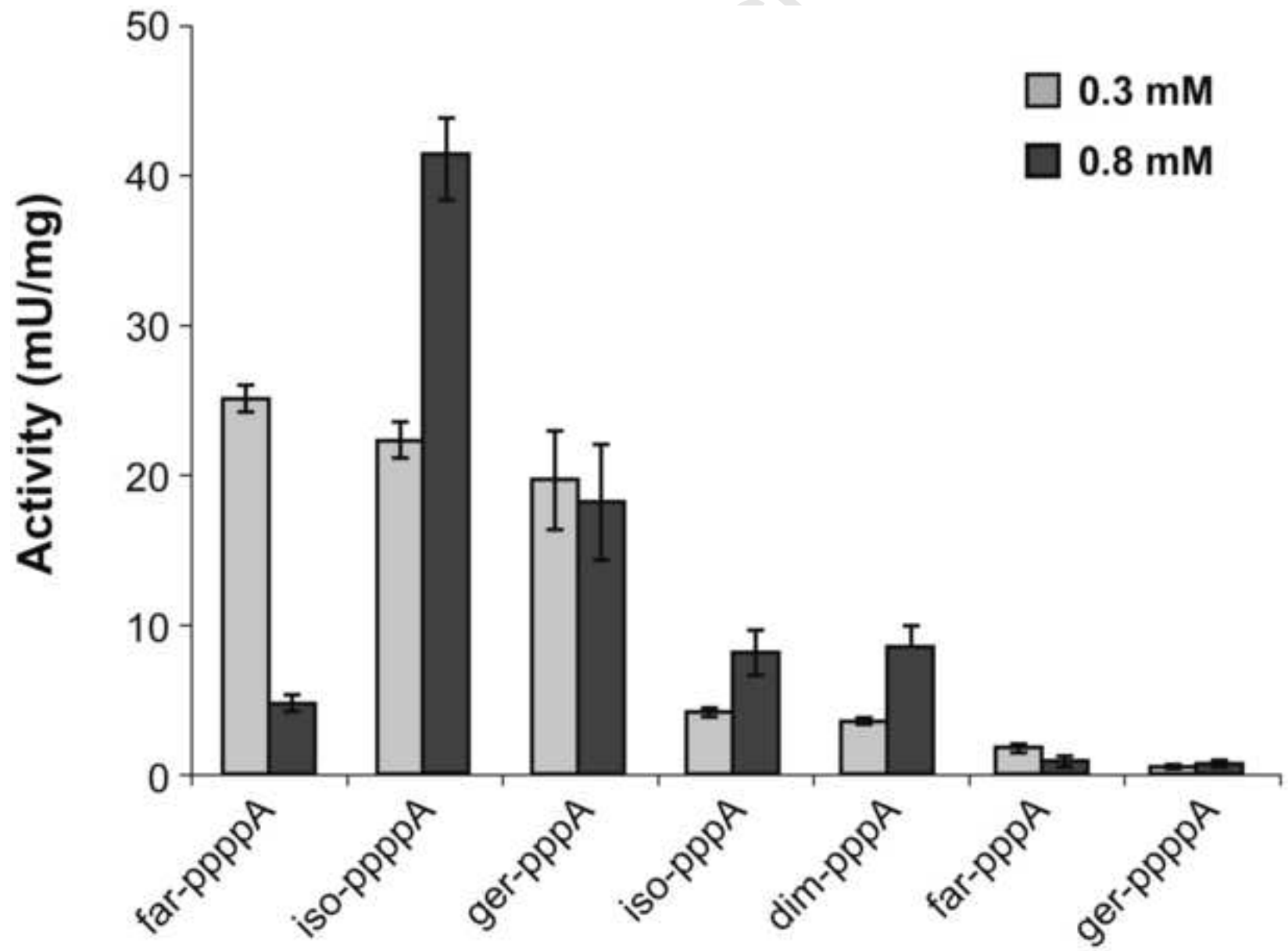


Figure 8

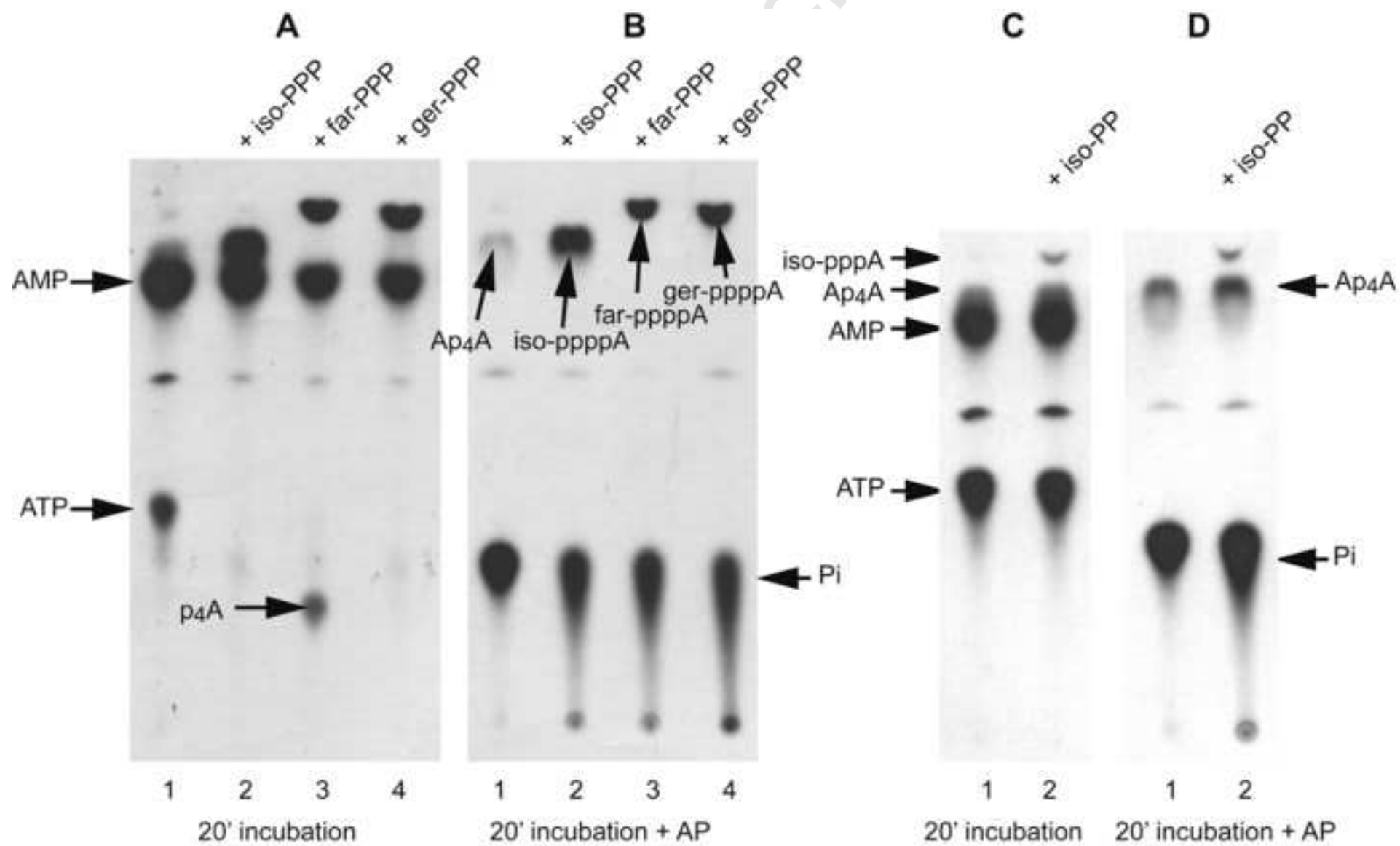


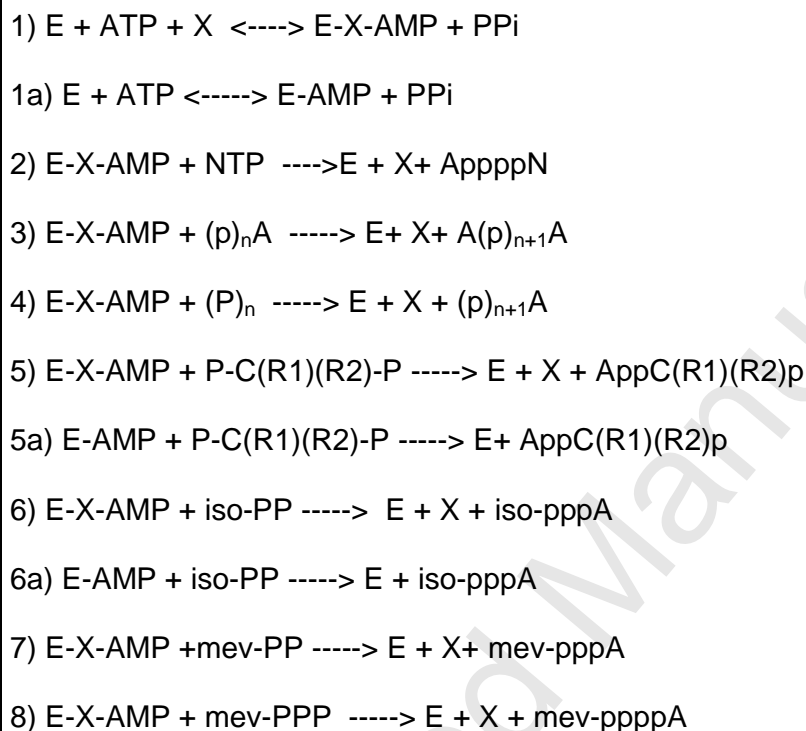
Fig.9

Mevalonate derivatives containing a terminal pyrophosphate form a new family of compounds catalyzed by ligases. Potential relationship with bisphosphonates



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Table 1 -- Reaction models catalyzed by ligases



X, a co-substrate of a ligase; NTP, a nucleoside triphosphate; $(p)_nA$, adenosine 5'-polyphosphate; $(P)_n$, a polyphosphate; P-C(R1)(R2)-P, a bisphosphonate; iso-PP, isopentenyl diphosphate; meV-PP and meV-PPP, components of the mevalonate pathway with a terminal pyrophosphate or triphosphate, respectively.

Table 2. V_{max} , K_m and K_{cat} values of T4 RNA ligase towards substrates of the mevalonate pathway

Substrate	V_{max} (mU/mg)	K_m (M)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ M ⁻¹)
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Farnesyl triphosphate	16.0±1.5	0.07±0.01 x10 ⁻³	11.6x10 ⁻³	166
Geranyl triphosphate	16.6±0.6	0.23±0.02 x10 ⁻³	12.0x10 ⁻³	52.2
Farnesyl diphosphate	4.0±0.5	0.24±0.02 x10 ⁻³	2.9x10 ⁻³	12.1
Geranyl diphosphate	3.4±0.1	0.29±0.06 x10 ⁻³	2.5x10 ⁻³	8.6
Isopentenyl triphosphate	33.5±5.1	3.6±0.5 x10 ⁻³	24.0x10 ⁻³	6.7
Dimethylallyl diphosphate	3.7±0.4	0.86±0.34 x10 ⁻³	2.7x10 ⁻³	3.1
Isopentenyl diphosphate	6.2±0.3	4.9±1.4 x10 ⁻³	4.5x10 ⁻³	0.9

Table 3. V_{max} , K_m and K_{cat} values of T4 DNA ligase towards substrates of the mevalonate pathway

Substrate	V_{max} (mU/mg)	K_m (M)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ M ⁻¹)
Farnesyl triphosphate	36.7±1.5	0.15±0.03 x10 ⁻³	34.9x10 ⁻³	232
Isopentenyl triphosphate	76.7±3.0	0.63±0.20 x10 ⁻³	72.9x10 ⁻³	116
Geranyl triphosphate	33.6±5.7	0.31±0.07 x10 ⁻³	31.9x10 ⁻³	103
Farnesyl diphosphate	2.9±0.4	0.05±0.01 x10 ⁻³	2.8x10 ⁻³	56
Isopentenyl diphosphate	33.3±10.6	2.3±0.93 x10 ⁻³	31.6x10 ⁻³	13.8
Dimethylallyl diphosphate	51.8±1.5	4.0±0.2 x10 ⁻³	49.2x10 ⁻³	12.3
Geranyl diphosphate	0.85±0.03	0.21±0.02 x10 ⁻³	0.8x10 ⁻³	3.8