The *pur6* gene of the puromycin biosynthetic gene cluster from *Streptomyces alboniger* encodes a tyrosinyl-aminonucleoside synthetase

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Received 29 July 2004; revised 14 September 2004; accepted 14 September 2004

Available online 22 October 2004

Edited by Stuart Ferguson

Abstract The *pur6* gene of the puromycin biosynthetic gene (*pur*) cluster from *Streptomyces alboniger* is shown to be essential for puromycin biosynthesis. Cell lysates from this mycelial bacterium were active in linking L-tyrosine to both 3′-amino-3′-deoxyadenosine and N^6,N^6-dimethyl-3′-amino-3′-deoxyadenosine with a peptide-like bond. Identical reactions were performed by cell lysates from *Streptomyces lividans* or *Escherichia coli* transformants that expressed *pur6* from a variety of plasmid constructs. Physicochemical and biochemical analyses suggested that their products were tridemethyl puromycin and *O*-demethylpuromycin, respectively. Therefore, it appears that Pur6 is the tyrosinyl-aminonucleoside synthetase of the puromycin biosynthetic pathway.

Keywords: *pur* cluster; *pur6* gene; Tyrosinyl-aminonucleoside synthetase; Puromycin biosynthesis; *Streptomyces*

1. Introduction

Puromycin is an aminonucleoside antibiotic, which is produced by *Streptomyces alboniger* (for a review, see [1]). Its biosynthetic pathway in this mycelial bacterium has been the subject of a variety of studies [2–8]. The puromycin biosynthetic gene (*pur*) cluster is within a 13.5-kb DNA fragment that has been successfully expressed in the heterologous hosts *Streptomyces lividans* and *Streptomyces griseofuscus* [9]. It contains 10 open reading frames of which those involved in puromycin biosynthesis appear to be transcribed in a single polycistronic mRNA [9]. Of these, the products of *napH*, *pur7*, *pur10*, *pac*, *dmpM* and *pur8* have been well or partially characterized [3–8]. Sequence comparisons suggested specific functions for the products of the remaining genes (*pur3*, *4*, *5* and *6*) [8]. Thus, Pur3 would be a phosphatase, Pur4 a putative aminotransferase, Pur5 a *N*-methyltransferase and Pur6 a tyrosinyl-nucleoside synthetase. These findings allowed proposal of a puromycin biosynthetic pathway with ATP as a substrate [10], which was later slightly modified [7]. ATP would be dehydrogenated by Pur10 (an NAD-dependent ATP dehydrogenase) to produce 3′-keto-3′-dehydroATP [8], which would generate 3′-amino-3′-dATP by the action of Pur4. This latter compound, which is a strong inhibitor of DNA-dependent RNA polymerases [11], is inactivated by Pur7, a nudix pyrophosphohydrolase, to produce 3′-amino-3′-dAMP [7]. At this stage, dephosphorylation at 5′ takes place by the action of the monophosphatase Pur3 (P. Barrado et al., unpublished results). The –COOH group of tyrosine would be then linked to 3′-amino-3′-dA by Pur6 ([10] and this work). It was proposed that tridemethylpuromycin (TDMP) would be *N*-acetylated by the puromycin *N*-acetyltransferase (Pac) enzyme, an *N*-acetyltransferase [5], to produce *N*-acetyl-TDMP. This intermediate would be dimethylated at N^6 by a *N*-methyltransferase (very likely Pur5) and then O-methylated by DmpM, an ODMP (*O*-demethylpuromycin)-O-methyltransferase [3,5,10]. This would yield the last precursor, the biologically inactive *N*-acetylpuromycin, which appears to be secreted and then hydrolyzed by NapH to produce the active antibiotic [7].

The putative tyrosinyl-nucleoside synthetase reaction catalyzed by Pur6 would be ATP-dependent. If so, one would expect certain similarities of Pur6 with non-ribosomal peptide synthetases or aminoacyl-tRNA synthetases; however, these were not found [10]. Its putative activity was suggested by elimination. An activity of these characteristics would be required in the biosynthetic pathway of puromycin and no other gene product from *pur* was a likely candidate, since either their functions were known or they were highly similar to proteins with different functions [10]. This work shows that indeed *pur6* is an essential gene of the *pur* cluster and suggests that it encodes a tyrosinyl-nucleoside synthetase, which would produce an intermediate containing the puromycin carbon backbone.

2. Materials and methods

2.1. Strains, media, plasmids, DNA methodology and chemicals

Streptomyces alboniger ATCC12461 [12], *S. lividans* 66 (1326) [13] and the *E. coli* DH5α [14] or BL21(DE3)pLysE [15] were previously

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Abbreviations: dA, deoxyadenosine; ODMP, *O*-demethylpuromycin; Pac, puromycin *N*-acetyltransferase; puromycin aminonucleoside, N^6,N^6-dimethyl-3′-amino-3′-deoxyadenosine; ORF, open reading frame; TDMP, tridemethylpuromycin

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doi:10.1016/j.febslet.2004.09.087

described in the indicated references. *Streptomyces* vectors were pIJ702 [16], pIJ6021 [17] and pGM9 [18] and those from *E. coli* were pBluescript SK(−) (Stratagene), pRSETb [19] and pJOE2775 [20]. Plasmid pFV5 is a pIJ702 derivative containing the *pac* gene from *pur* [21]. *Streptomyces* was grown in solid medium R5 [13], and YEME, containing 5 mM $MgSO_4$, and S media for liquid cultures [9]. When required, thiostrepton (10 μg/ml), hygromycin B (200 μg/ml), neomycin B (13 μg/ml), kanamycin (50 μg/ml), ampicillin (100 μg/ml), or chloramphenicol (34 μg/ml) was added at the indicated final concentration. DNA methodology was applied as described [22].

Puromycin aminonucleoside and puromycin were obtained from Sigma. 3′-Amino-3′-dATP, L-[U-^{14}C]tyrosine (448 mCi/mmol) and [^{3}H]acetyl-CoA (5.2 Ci/mmol) were from Amersham. 3′-Amino-3′-dA was obtained from *Helminthosporium* sp. [23]. ODMP and acetyl-ODMP were chemically synthesized [5]. 3′-Amino-3′-dAMP was obtained as described [24]. 5′-P-puromycin aminonucleoside was obtained by chemical phosphorylation (TriLink BioTechnologies, San Diego, CA).

2.2. Preparation of a pur6 deletion mutant

From a *Nru*I–*Eco*RI fragment of *pur* (nucleotides 2933–6314) [10], an *Acc*I–*Sty*I internal fragment (nucleotides 333–1687) of the *pur6* coding sequence (58%) [10] was replaced by a *hyg* gene, which contained the promoter and coding sequence and lacked the transcription terminator signal. The resulting fragment was inserted in the *Xho*I–*Xba*I thermosensitive replicon fragment of pGM9. The resulting pSDP6.2 was transformed into *S. alboniger* [18]. Neomycin B-sensitive, hygromycin B-resistant colonies were selected. Southern blot analyses permitted to characterize several correct mutants (data not shown).

2.3. Expression of Pur6 in Streptomyces

To express Pur6 in *S. lividans*, a 2.7-kb *Not*I–*Bgl*II fragment (nucleotides 3524–6204) [10] from *pur*, which contained an intact *pur6* gene, was inserted in the *Bgl*II replicon fragment of pIJ702. One of the resulting construct (pSEXP6.2), which contained *pur6* in the same orientation than the *mel* promoter of pIJ702, was isolated upon transformation into *S. lividans*. In addition, pur6 was included under the thiostrepton-inducible *tipA* promoter of pIJ6021 resulting in construct pSEXP6.3.

To prepare cell lysates, *S. alboniger* and *S. lividans* strains were grown in S and YEME medium, respectively. Antibiotics were added as required. Cell mycelia from 12 ml cultures were resuspended in 0.5 ml of 300 mM NaCl, 50 mM KH_2PO_4, pH 8.0, and sonicated 3 times for 10 s at 15 micron at 4 °C. Lysates were spun at 15 000 ×*g* for 10 min and the resulting supernatants were used immediately or maintained at −70 °C. Proteins were analyzed by SDS–PAGE [22].

2.4. Enzymatic assays

Unless otherwise indicated, a Pur6 assay (20 μl), as deduced from an optimization process, contained 1 mM $MgCl_2$, 1 mM ATP, 1 mM of the different substrates assayed, 0.1 mM L-[U-^{14}C]tyrosine (50 mCi/mmol), 50 mM Tris–HCl, pH 8.8, and 8–35 μg protein (the optimal one for the relevant lysate) from either *Streptomyces* or *E. coli* lysates. Unless otherwise indicated, reactions were incubated for 30 min at 30 °C and then stopped by heating at 85 °C for 5 min. These were found to be the optimal conditions. Samples (0.5 μl) were analyzed using TLC in four different systems. Systems 1 and 2: PEI-Cellulose F (Merck) and 1 M LiCl or 0.1 M ammonium acetate, acetonitrile (1:2), respectively. Systems 3 and 4: cellulose F (Merck) and 0.1 M ammonium acetate, acetonitrile (80:20) or 1-butanol, acetic acid, water (12:3:5), respectively. Dried sheets were exposed for 5–15 h on a Fuji Bas Imaging plate MP2040S and analyzed by Fujifilm BAS-1500. Analysis and quantification were performed with the aid of TINA 2.0 program. Protein was quantified with the Bio-Rad assay kit.

For sequential Pur6 and Pac assays, Pur6 reactions were carried out in the presence of *S. lividans* (pSEXP6.2 or pSEXP6.3) lysates and L-[^{14}C]tyrosine and then inactivated by heating at 85 °C for 5 min. They were then made up 10% glycerol (v/v), 2 mM EDTA (to complex Mg^{2+} ions), 0.2 mM acetyl-CoA and 50 mM Tris–HCl, pH 8.5. 10 μl of a *S. lividans* (pIJ702) or *S. lividans* (pFV5) [21] lysate was then added to a final volume of 50 μl. Reactions were incubated at 30 °C for 30 min and analyzed by TLC as referred. When Pur6 reactions were performed with unlabeled 0.1 mM L-tyrosine, assays were performed as referred except that 1.6 nM [^{3}H]acetyl-CoA was used. Puromycin was quantified in culture filtrates by the Pac assay [5].

2.5. Expression of Pur6 in E. coli

A 2.4-kb *Mse*I–*Bgl*II (3768-6204) [10], which contains the complete *pur6* sequence except its three initial codons, was blunt-ended with the Klenow enzyme and inserted in the *Pvu*II replicon fragment of the IPTG- and the rhamnose-inducible expression vectors pRSETb and pJOE2775, respectively. The last plasmid was expressed in *E. coli* JM109. Expression and attempts of purification of Pur6 were carried out as described [15,19].

3. Results and discussion

3.1. Pur6 activity in different cell extracts

Putatively, Pur6 should catalyze a peptide-like amide bond between the –COOH group of tyrosine and the 3′-amino group of 3′-amino-3′-dA or a close intermediate [10]. This reaction would require an activation of tyrosine, which could be achieved by means of ATP and perhaps a divalent cation. Therefore, Pur6 activity was initially assayed in a cell lysate from *S. lividans* (pSEXP6.2), in the presence of either 3′-amino-3′dA, puromycin aminonucleoside, or adenosine. The results showed the modification of the first and second substrate, but not of the third one. These reactions were ATP- and Mg^{2+}-dependent (Fig. 1 and data not shown). The product of the reaction in the presence of 3′-amino-3′-dA was thought to be TDMP, whereas that with puromycin aminonucleoside had identical R_f to synthetic ODMP in four different TLC systems (Table 1), therefore suggesting that this was the reaction product. The reaction was linear for the initial 5 min. After 25–30 min of incubation, a plateau was reached. The final yield of the product was lower than 5% of the initial L-[^{14}C]tyrosine present in the reaction (data not shown). Since 3′-amino-3′-dAMP and 5′-P-puromycin aminonucleoside were not substrates of Pur6, they most likely did not appear as biosynthetic intermediates, which clarifies earlier speculations [10]. Neither cordycepin (3′-deoxyadenosine) nor the aminonucleoside antibiotic A201A that contains the 3′-amino-3′-dA moiety of puromycin were substrates of Pur6 (data not shown).

Pur6 activity and puromycin production were assayed in samples taken at different times during the growth curve of a variety of *Streptomyces* strains. In *S. alboniger*, both puromycin and Pur6 activity were initially detected at the middle of the log phase and reached a maximum at 50–60 h of growth

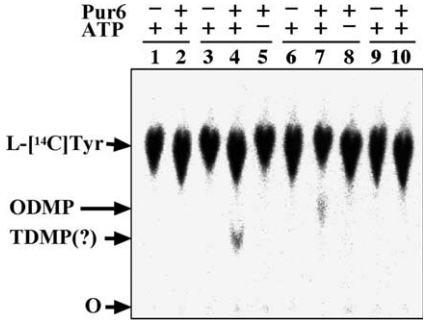

Fig. 1. Pur6 assays with cell lysates from *S. lividans*. Assays were carried out in the presence of L-[^{14}C]tyrosine, the indicated substrates (all at 1 mM) and *S. lividans* (pSEXP6.2 or pIJ702) lysates. TLC was performed with system 1. Lanes 1–2, no substrate. Lanes 3–5, 3′-amino-3′-dA. Lanes 6–8, puromycin aminonucleoside. Lanes 9–10, adenosine. O, origin of the TLC. + and − indicate presence or absence of Pur6 and/or ATP.

Table 1
R_f values of several compounds in four TLC systems

Compound	TLC Systems			
	1	2	3	4
3′-Amino-3′-dA[a]	0.36	0.44	0.53	0.35
Puromycin aminonucleoside[a]	0.47	0.81	0.72	0.51
L-Tyrosine[b]	0.63	0.48	0.73	0.39
ATP[a]	0.28	0.06	0.93	0.10
ADP[a]	0.56	0.16	0.90	0.13
AMP[a]	0.67	0.49	0.97	0.20
TDMP[b,c]	0.27	0.67	0.54	0.39
ODMP[a,b,d,e]	0.37	0.88	0.73	0.63
N-Acetyl TDMP[b,f]	0.38	0.86	0.63	0.72
N-Acetyl ODMP[a,b,e,f]	0.52	0.96	0.77	0.93
Puromycin[a]	0.66	0.96	0.75	0.78
Acetyl CoA[b]	0.93	0.78	0.98	0.61
N-Acetyl puromycin[b,e]	0.54	1	0.81	0.94

[a] Detected with UV light at 254 nm.
[b] Detected as a radioactive product.
[c] Product of a Pur6 reaction with L-[^{14}C]tyrosine and 3′-amino-3′-dA as substrates.
[d] Product of a Pur6 reaction with L-[^{14}C]tyrosine and puromycin aminonucleoside as substrates.
[e] Chemically synthesized.
[f] As [3] and [4] but *N*-acetylated with Pac.

(Fig. 2). As expected, no activity was detected in *S. alboniger* Δ*pur6* (pIJ702) lysates, whereas it was reinstated in a time-dependent manner in *S. alboniger* Δ*pur6* (pSEXP6.2). Similar results were obtained with lysates from a thiostrepton-induced *S. lividans* (pSEXP6.3), which abundantly expressed a protein of a size similar to that of Pur6 (84 kDa) (Fig. 3). However, all attempts to purify Pur6 from the different *Streptomyces* strains that expressed it were unsuccessful. Apparently, the activity was destroyed by ultracentrifugation on glycerol gradients or by DEAE-cellulose or DEAE-Sepharose CL-6B column chromatography. It only supported a filtration cycle through a PD-10 Sephadex G-25 column. Nevertheless, this preparation lost its activity after 24 h at 4 °C or by a cycle of freezing and thawing (data not shown). These results suggested that under the conditions used, Pur6 was highly labile. Further purification attempts were carried out in *E. coli* by adding a six His tale to Pur6 in the expression vectors pRSETb and pJOE2775. Most of the expressed fusion Pur6 protein formed inclusion bodies, a phenomenon that was not alleviated by varying

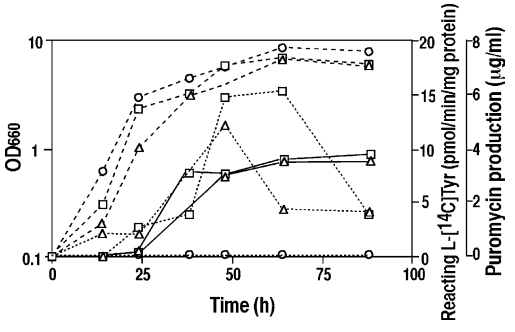

Fig. 2. Expression of Pur6 in different strains. Culture filtrates were obtained at the indicated times from *S. alboniger* (□) and *S. alboniger* Δ*pur6* transformed with either pIJ702 (○) or pSEXP6.2 (△). At the indicated times puromycin (–) was quantified by the Pac assay. Cell lysates were obtained and their Pur6 activity (···) was assayed in the presence of 3′-amino-3′-dA. OD_{660}(- - -).

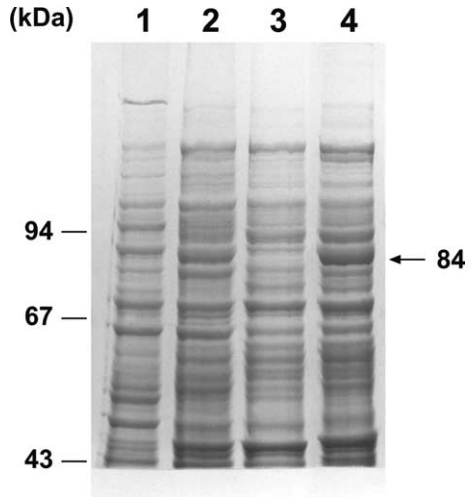

Fig. 3. Electrophoretic analysis of cell lysates. Cell lysates of *S. lividans* transformed with pIJ702 (1), pSEXP6.2 (2), pIJ6021 (3) or pSEXP6.3 (4) were subjected to SDS–PAGE (7%) and stained with Coomasie Blue. The positions of molecular mass markers are indicated at the left.

culture conditions or using different *E. coli* strains (data not shown). Although the minimal amount of soluble Pur6 that accumulated in the crude lysates permitted to detect clearly its activity, further purification attempts through Ni–NTA columns failed to recover active enzyme.

3.2. The pur6 gene is essential for puromycin biosynthesis

Six *S. alboniger* Δ*pur6* mutants were obtained (see Section 2). None of them produced puromycin (Fig. 2 and data not shown). Therefore, these findings indicate that *pur6* is an essential gene of the *pur* cluster. Construct pSEXP6.2, which carries *pur6*, was introduced in several *S. alboniger* Δ*pur6* mutants. In all cases, pSEXP6.2 rescued puromycin biosynthesis (an example is presented in Fig. 2), which confirms that *pur6* is essential for puromycin production in *S. alboniger*.

3.3. Characterization of the Pur6 reaction products

The Pac enzyme of the puromycin biosynthetic pathway N-acetylates puromycin, and its product, *N*-acetylpuromycin, is biologically inactive. Pac is a highly specific enzyme, which only recognizes compounds with the puromycin carbon backbone like ODMP and chriscandin [5]. Therefore, it should be expected that the products of Pur6 reactions with either 3′-amino-3′-dA or puromycin aminonucleoside (the putative TDMP and ODMP, respectively) were *N*-acetylated by Pac. To examine this possibility, two different assays were performed. In the first one, Pur6 reactions were carried out with L-[^{14}C]tyrosine and either 3′-amino-3′-dA or puromycin aminonucleoside. Then their products were reacted, in the presence of unlabeled acetyl-coenzyme A, with Pac. In the second one, Pur6 reactions were carried out with unlabeled L-tyrosine, whereas Pac reactions were performed with [^{3}H]acetyl-CoA. In both cases, novel radioactive products with similar R_f appeared, which were absent in the control reactions lacking Pur6 or Pac (Fig. 4). In addition, the spots identified as *N*-acetyl-ODMP had similar R_f than synthetic *N*-acetyl-ODMP in the four TLC systems (Table 1). These findings suggest that the products synthesized in Pur6 reactions are *N*-acetylated by Pac. Given the high specificity of this enzyme and the mobility

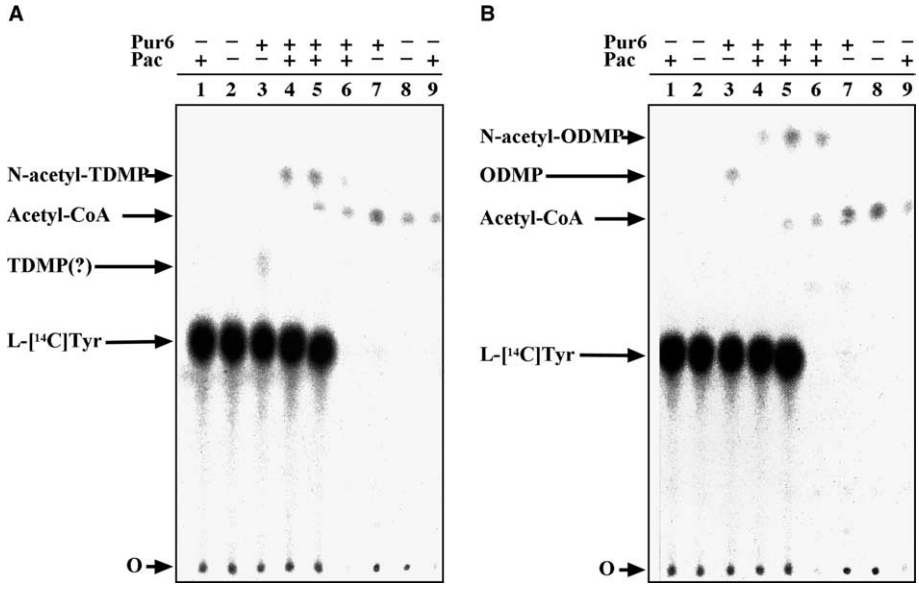

Fig. 4. Sequential Pur6 and Pac assays. Pur6 reactions were performed with either 3′-amino-3′-dA (A) or puromycin aminonucleoside (B). TLC was performed with system 2. R_fs are given in Table 1. Lanes 1–4, Pur6 and Pac reactions were performed in the presence of L-[^{14}C]tyrosine and unlabeled acetyl-CoA, respectively. Lanes 6–9, Pur6 and Pac reactions were performed with unlabeled L-tyrosine and [^{3}H]acetyl-CoA, respectively. An equal mixture of samples from reactions 4 and 6 was spotted on line 5. + and − indicate presence or absence of Pur6 and/or Pac, respectively. O, origin of the TLC. Cell lysates were from *S. lividans* (pSEXP6.3).

of the reaction products, these findings strongly suggest that Pur6 catalyzes the incorporation of L-tyrosine on both 3′-amino-3′-dA and puromycin aminonucleoside producing TDMP or ODMP, respectively (Fig. 5). Furthermore, TDMP was detected in samples of puromycin, which suggested that this compound is an intermediate of the pathway. This suggestion was reinforced by the presence of N^6,*O*-didemethyl-puromycin and ODMP, the putative consecutive intermediates, in the analyzed puromycin samples [4]. Although Pur6 catalyzes both reactions with apparently similar kinetics (data not shown), the experimental conditions used in this work did not permit to determine the K_m values for both substrates, which could helped to identify the correct reaction in the puromycin biochemical pathway [5]. The mechanism of the reaction catalyzed by Pur6 would be reminiscent of that used by the non-ribosomal peptide synthetases. Thus, the Pur6 reaction requires ATP and Mg^{2+} ions, as it is the general case with peptide synthetases. However, from an evolutionary point of view, Pur6 does not present clear similarities with members of this protein family. Indeed, it lacks all their conserved domain and it does not present similarities to other proteins, including aminoacyl-tRNA synthetases. In fact, its novel catalyzed reaction is quite separate from those catalyzed by these groups of proteins, which could help to explain their dissimilarity. In any case, by similarity with these synthetases, the fate of the ATP during reaction might be AMP+PPi. However, there was no indication on this possibility on the TLC plates nor further work on this subject was performed. There are other known natural aminonucleoside antibiotics produced by fungi (homocitrullylaminoadenosine, lisylaminoadenosine and chrisCandine) that contain a 3′-amido linkage similar to that of puromycin. Presumably, they contain enzymes similar to Pur6, although they have not yet been identified.

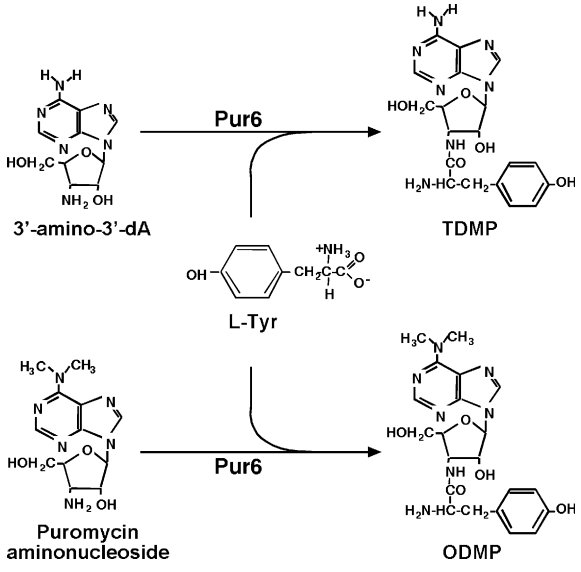

Fig. 5. Schematic view of possible reactions catalyzed by Pur6.

Acknowledgements: We thank A. Martín for technical assistance. This work was supported by grants BIO1999-0959 and BIO2003-03314 of CICYT. We also thank the Fundación Ramón Areces for an institutional grant to the CBMSO.

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