Identification and structural characterization of O-β-ribosyl-(1″→2′)-adenosine-5″-phosphate in yeast methionine initiator tRNA

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ABSTRACT

We report in this paper on the complete structure determination of the modified nucleotide A*, now called Ar(p), that was previously identified in yeast methionine initiator tRNA as an isomeric form of O-ribosyl-adenosine bearing an additional phosphoryl-monoester group on its ribose$_2$ moiety. By using the chemical procedure of periodate oxidation and subsequent β-elimination with cyclohexylamine on mono- and dinucleotides containing Ar(p), we characterized the location of the phosphate group on the C-5″ of the ribose$_2$ moiety, and the linkage between the two riboses as a (1″→2′)-glycosidic bond. Since the structural difference between phosphatase treated Ar(p) and authentic O-α-ribosyl-(1″→2′)-adenosine from poly(ADP-Ribose) was previously assigned to an isomeric difference in the ribose$_2$-ribose$_1$ linkage, the (1″→2′)-glycosidic bond of Ar(p) was deduced to have a β-spatial configuration. Thus, final chemical structure for Ar(p) at the position 64 in yeast initiator tRNA^{Met} has been established as O-β-ribosyl-(1″→2′)-adenosine-5″-phosphate. This nucleotide is linked by a 3′,5′-phosphodiester bond to G at the position 65.

INTRODUCTION

A minor modified nucleotide A* previously located at the position 64 in yeast methionine initiator tRNA (yeast initiator tRNA^{Met}) (1) has been lately identified by our group as an isomeric form of O-ribosyl-adenosine (2). Our studies by high performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS) indicated a slight difference of chemical structure between phosphatase treated [A*] of yeast initiator tRNA^{Met} and authentic O-α-ribofuranosyl-(1″→2′)-adenosine (Ado-Rib) that we isolated from poly(adenosine-diphosphate-ribose), i.e. poly(ADP-Rib).

According to our electron impact−mass spectrometry (EI-MS) data (2), this structural difference between A* and Ado-Rib was assigned to an isomeric difference in the ribose$_2$-ribose$_1$ linkage. In the Ado-Rib residues of poly(ADP-Rib), the linkage between the two riboses was clearly characterized as an α(1″→2′)-glycosidic bond (3−7). Thus, ribose$_2$ and ribose$_1$ of the isomer A* nucleotide from yeast initiator tRNA^{Met} could be linked through either β(1″→2′), or α(1″→3′), or β(1″→3′)-glycosidic bond. Our studies on A* derivatives brought also evidence for the presence of an additional phosphorylmonoester group located on the ribose$_2$ of this new modified nucleotide.

Because of its close structure relationship with Ado(P)-Rib(P) isolated from poly(ADP-Rib), and to distinguish it from this compound, the A* nucleotide of yeast initiator tRNA^{Met} will be called by the abbreviation Ar(p), while the corresponding phosphatase treated [A*] nucleoside will be called Ar.

We report in this paper our investigations on the final structural determination of this phosphorylated O-ribosyl-adenosine nucleotide located at the position 64 in yeast initiator tRNA^{Met}. By using the chemical procedure of periodate oxidation and subsequent β-elimination with cyclohexylamine on several mono- and dinucleotides containing Ar(p), we have determined the location of the additional free monophosphate group on the ribose$_2$ moiety, as well as the carbons involved in the glycosidic bond between the two ribose moieties, and the spatial conformation of this glycosidic bond.

MATERIALS AND METHODS

Isolation of Ar(p) containing mono- and dinucleotides

DEAE-cellulose column chromatography. Partially purified yeast initiator tRNA^{Met} was hydrolyzed by pancreatic RNase (Worthinghton, USA) (8). The resulting oligonucleotide mixture was chromatographed on fibrous DEAE-cellulose from Schleicher and Schüll (Dassel, GFR). Elution was performed with a linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl pH 8. The hexanucleotidic fraction containing the tetranucleotide GpAr(p)pGpCp (2) was collected and desalted as previously described (8,9).

Further hydrolyses of this hexanucleotidic fraction were carried out as previously described (8,10) with T2-RNase or nuclease P1. The corresponding digests contained Ar(p)pGp or pAr(p)pG,

respectively. These two dinucleotides eluted in the tetranucleotidic fractions when chromatographed on a DEAE-cellulose column. They were further isolated from contaminating tetranucleotides by thin layer chromatography (TLC) as described below.

Thin layer chromatography. Thin layer chromatography (TLC) on cellulose plates (F1440 or G1440 from Schleicher and Schüll, Dassel, GFR), was used to isolate Ar(p)pGp or pAr(p)pG from the tetranucleotidic fractions. The following solvents (10) were used :

A : isobutyric acid-25 % NH_4OH-H_2O (50:1.1:5.9, v/v/v)
B : HCl-isopropanol-H_2O (15:70:15, v/v/v)

HPLC-UV analysis. The Ar(p)pGp and pAr(p)pG dinucleotides isolated by TLC were purified and desalted by HPLC using the analytical procedure previously described for the isolation of unknown nucleotides and nucleosides (2,11). Each collected fraction was evaporated to dryness under vacuum at room temperature in a Savant Speed Concentrator (Savant Instrument, Inc., Farmingdale, USA).

The Ar(p) and pAr(p) mononucleotides were then isolated by HPLC from snake venom phosphodiesterase digests (2) of Ar(p)pGp and pAr(p)pG dinucleotides, respectively.

Authentic O-α-ribosyl-(1″→2′)-adenosine-5′,5″-bis(phosphate), i.e. Ado(P)-Rib(P), was isolated by HPLC from a snake venom phosphodiesterase digest of biosynthetic poly(ADP-Rib) as previously described (2,12,13). This mononucleotide served as the reference isomer for the structural determination of Ar(p).

Aliquots of these mono- and dinucleotides were then submitted to the chemical procedure of periodate oxidation and subsequent β-elimination, as described below.

Periodate oxidation−β-elimination procedure

The periodate oxidation−β-elimination procedure (P.O.-βE. procedure) described by Keith and Gilham (14) for the stepwise degradation of polyribonucleotides was adapted to the chemical degradation of Ar(p) containing mono- and dinucleotides isolated from yeast initiator $tRNA^{Met}$.

The mononucleotides or dinucleotides (ca. 100 nmol) in 150 µl of distilled water were treated at O°C for 60 min with 20 µl of 0.2 M sodium periodate (Sigma, St Louis, USA), followed by 20 µl of O.4 M rhamnose (Sigma, St Louis, USA) at 0°C for 30 min in order to neutralize the remaining sodium periodate. To the mixture, were then added 60 µl of 2 M cyclohexylamine hydrochloride, and the solution was incubated at 45°C for 90 min.

The products present in the final solution were analyzed by HPLC.

RESULTS

Study of Ar(p) derivatives by HPLC

The high selectivity and efficiency of the reversed phase column is essential for the separation by HPLC of a large number of modified nucleosides and nucleotides. One of the major problems for the analytical biochemist is to obtain the reference molecules.

We have therefore determined and standardized the chromatographic retention times of the appropriate compilation of compounds implicated in the present study. For the nucleotides, nucleosides and nucleic bases involved as oxidation products of P.O.-βE. of Ar(p) derivatives, the HPLC retention times were determined using standard compounds dissolved, either in aqueous solutions, or in P.O.-βE. reaction mixture. The mononucleotides which eluted during the first 20 minutes of the HPLC run were retained much more on the chromatographic column when applied in P.O.-βE. reaction mixture than in aqueous solution (e.g. adenosine-5′-phosphate, guanosine-3′-phosphate, adenosine-3′,5′-bis(phosphate), Ado(P)-Rib(P), etc...). Such retention times increases were not observed for the nucleic bases which eluted during the first 20 minutes (adenine and guanine), or for the nucleosides, mononucleotides and dinucleotides which eluted after 20 minutes (e.g. adenosine, guanosine, adenosine-3′-phosphate, ApG, pAr(p)pG, etc...).

A Table, compiling the retention times of all the derivatives used in the present study, is available upon request, or will be added to all reprint requests.

Periodate oxidation−β-elimination on pAr(p) and Ar(p) mononucleotides

When applied to nucleosides or 5′-mononucleotides, the P.O.-βE. procedure leads to the liberation of the corresponding nucleic bases, whereas it is inefficient on 2′- or 3′-phosphate mononucleotides. Thus, the oxidation products obtained from adenosine-5′-phosphate and adenosine-3′-phosphate were identified by HPLC as adenine and intact adenosine-3′-phosphate, respectively.

The P.O.-βE. procedure was also efficient on reference Ado(P)-Rib(P) from poly(ADP-Rib) as well as on pAr(p) mononucleotide from yeast initiator $tRNA^{Met}$, leading in both cases to adenosine-5′-phosphate, i.e. removing the phosphoryl-$ribose_2$ moiety from each ribosyl-adenosine isomer. This removal of phosphoryl-$ribose_2$ moiety was confirmed on phosphatase untreated Ar(p), which led to adenosine nucleoside after P.O.-βE. treatment.

From these results (*Fig. 1*), one can conclude that the hydroxyl groups on carbons 2″ and 3″ of $ribose_2$ moiety in pAr(p) and Ar(p) mononucleotides are free. Thus, the additional phosphorylmonoester group can be only located on the carbon 5″.

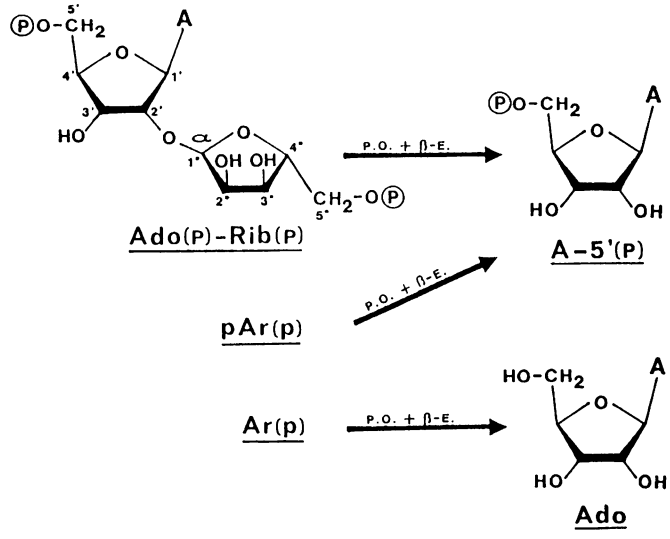

Fig. 1. Summarized results of P.O.-βE. procedure applied to pAr(p) and Ar(p) mononucleotides isolated from yeast initiator $tRNA^{Met}$, and Ado(P)-Rib(P) isolated from poly(ADP-Rib). The nucleic compounds resulting from these oxidative degradations were identified as adenosine-5′-phosphate and adenosine by their HPLC retention times (13.4 min and 38.6 min, respectively), and UV-absorption spectra (results not shown).

Periodate oxidation–β-elimination on Ar(p) containing dinucleotides

The P.O.-βE. chemical procedure applied on any dinucleotide N_1pN_2 should consist of a degradative cycle involving, i) the periodate oxidation of the 2',3'-terminal cis-diol group, ii) the cleavage of the 3',5'-phosphodiester bond (β-elimination reaction) leading to a mixture of 3'-phosphorylated 5'-terminal nucleoside (N_1-3'p) and oxidized 3'-terminal nucleoside moiety (oxidized N_2), and iii) the subsequent conversion of the latter nucleoside fragment to the corresponding base. These chemical degradative properties of P.O.-βE. were initially confirmed on adenylyl-3',5'-guanosine standard dinucleotide (ApG) by leading to a mixture of adenosine-3'-phosphate and guanine.

The pAr(p)pG dinucleotide isolated from yeast initiator $tRNA^{Met}$ was submitted to P.O.-βE. chemical action. *Fig. 2a* shows the HPLC chromatogram of the oxidative products present in the final mixture. One of these products was easily identified as guanine by its HPLC retention time and UV-absorption spectrum. According to the chromatographic retention times and spectrometric properties of authentic adenosine-3',5'-bis(phosphate) and adenosine-2',5'-bis(phosphate), another oxidative compound was characterized as adenosine carrying two phosphorylmonoester groups located, either at the positions 3' and 5', or at the positions 2' and 5'. Thus, the chemical degradation of pAr(p)pG dinucleotide by P.O.-βE. was efficient for the removal of the phosphoryl-ribose$_2$ part of Ar(p) moiety, as already described above for Ar(p) and pAr(p) mononucleotides, and for the cleavage of the G nucleoside moiety with its subsequent conversion to guanine. However, since one phosphate group could not be located exactly on the released adenosine-diphosphate moiety (C-2' or C-3'), the carbon of Ar(p) involved in the phosphodiester bond between Ar(p) and G remained to be determined.

In order to characterize this phosphodiester bond, two additional experiments were carried out on Ar(p)pGp.

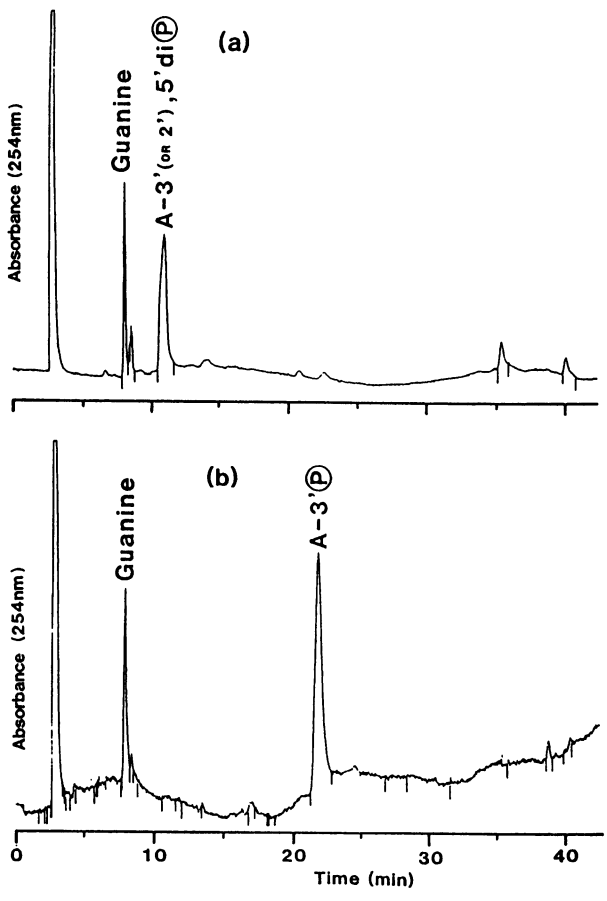

Fig. 2. HPLC chromatograms of the oxidative products resulting from the P.O.-βE. procedure applied: (a) to pAr(p)pG dinucleotide: the chromatographic peak eluted at 8.0 min retention time was identified as guanine, while the peak eluted at about 11 min could correspond either to adenosine-3',5'-diphosphate, or to adenosine-2',5'-diphosphate, (b) to Ar(p)pG dinucleotide obtained from nuclease P1 digestion of Ar(p)pGp dinucleotide: the peaks eluted at 8.0 min and 21.8 min were identified as guanine and adenosine-3'-phosphate, respectively.

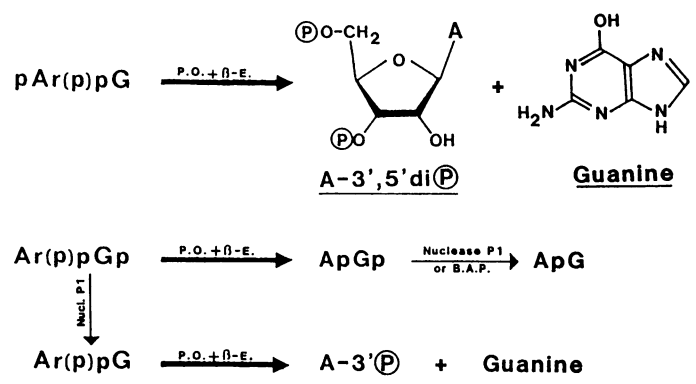

Fig. 3. Summarized results of P.O.-βE. procedure applied: i) to pAr(p)pG dinucleotide: identification of two oxidative products as adenosine-3'(or 2'),5'-diphosphate (HPLC retention times of about 11 min for both standard compounds), and guanine (8.0 min HPLC retention time), ii) to Ar(p)pGp dinucleotide: identification of one oxidative product as ApGp dinucleotide (36.4 min HPLC retention time), which yielded 3'-dephosphorylated ApG dinucleotide (44.8 min HPLC retention time) upon nuclease P1 or bacterial alkaline phosphatase treatment, iii) to Ar(p)pG dinucleotide resulting from nuclease P1 hydrolysis of Ar(p)pGp dinucleotide: identification of two oxidative products as adenosine-3'-phosphate (21.8 min HPLC retention time), and guanine (8.0 min HPLC retention time).

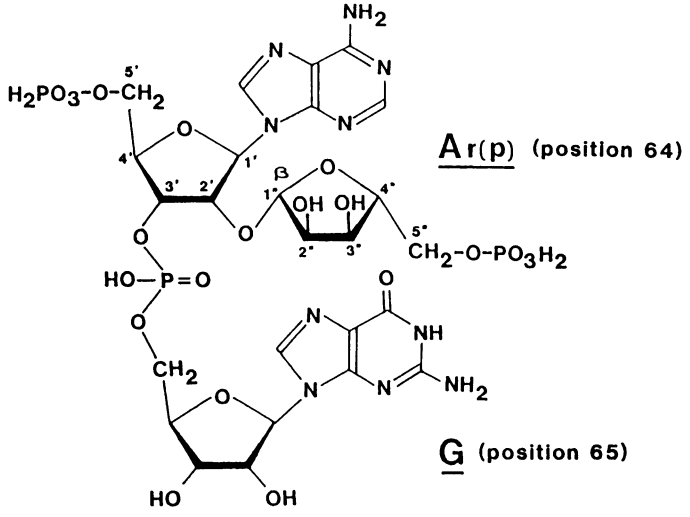

Fig. 4. Chemical structure of the dinucleotide pAr(p)pG isolated from nuclease P1 digestion of yeast initiator $tRNA^{Met}$ (ref. 2 and this paper). This dinucleotide consists of O-β-ribosyl-(1"→2')-adenosine-5',5"-bis(phosphate) located at the position 64 in the T-stem, and linked by a 3',5'-phosphodiester bond to guanosine at the position 65.

This dinucleotide was first submitted to P.O.-βE. procedure followed by bacterial alkaline phosphatase or nuclease P1 hydrolysis. The only oxidative product present in the final mixture was identified as adenylyl-3',5'-guanosine (ApG) by comparison of its HPLC retention time and UV-absorption spectrum with those of authentic standard ApG. From this result, we could conclude that Ar(p) nucleotide and G nucleotide were linked by a 3',5'-phosphodiester bond.

In the second experiment, nuclease P1 digestion of Ar(p)pGp dinucleotide led to 3'-dephosphorylated Ar(p)pG dinucleotide which yielded a mixture of two oxidative compounds upon P.O.-βE. treatment. These compounds were identified as adenosine-3'-phosphate and guanine by their retention times in HPLC (*Fig. 2b*) and their UV-absorption spectra. Since the released adenosine moiety was phosphorylated at the position 3', the bond between Ar(p) and G was confirmed as a 3',5'-phosphodiester bond.

The above results on the chemical degradation of Ar(p) containing dinucleotides are summarized in *Fig. 3*. They provide the evidence for the 3',5'-phosphodiester structure of the bond between the two nucleoside moieties in Ar(p)pG dinucleotide. Consequently, the linkage between $ribose_2$ and $ribose_1$ in Ar(p) nucleotide can be only a (1"→2')-glycosidic bond. These results also confirmed the location of the additional monophosphate group at the position 5" of $ribose_2$ moiety.

DISCUSSION AND CONCLUSION

In this paper, we bring the evidence that the $ribose_2$ moiety of O-ribosyl-adenosine in yeast initiator $tRNA^{Met}$ carries a phosphorylmonoester group located on its carbon 5". In addition, this $ribose_2$ is shown to be linked by a (1"→2')-glycosidic bond to the $ribose_1$ of adenosine moiety. According to the chiral center at C-1" of $ribose_2$, only two alternative configuration possibilities exist for the (1"→2')-glycosidic bond. This bond would be either in α-configuration, or in β-configuration. Since the α-configuration was rigorously demonstrated for Ado-Rib from poly(ADP-Rib) (3−7), and because Ar nucleoside from yeast initiator $tRNA^{Met}$ had the same EI-MS fragment ion-series as Ado-Rib, but behaved differently on TLC, GC and HPLC (2), $ribose_2$ and $ribose_1$ of Ar(p) nucleotide can only be linked through a β(1"→2')-glycosidic bond.

Previously announced nuclear magnetic resonance (NMR) studies (2) confirmed the (1"→2')-glycosidic linkage for Ado-Rib from poly(ADP-Rib), and for Ar from yeast initiator $tRNA^{Met}$, since the chemical shift for the 2'-hydroxyl proton was missing in both compounds (results not shown). In addition, the comparison between the NMR patterns of Ado-Rib and Ar showed that these compounds were essentially different since they displayed different patterns. However, the signals formed for Ar did not allow the rigorous direct determination of the (1"→2')-bond configuration, even if the order of the ribosyl 2"- and 3"-hydroxyl protons corresponded, as previously described (5), to that of a β anomer.

Thus, the final structure for Ar(p) in yeast initiator $tRNA^{Met}$ was deduced as O-β-ribosyl-(1"→2')-adenosine-5"-phosphate linked by a 3',5'-phosphodiester bond to G at the position 65 (*Fig. 4*).

Regarding the biosynthesis of this modified nucleotide, the biochemical pathway should consist in a post-transcriptional β(1"→2') ribosylation of adenosine residue at the position 64 in yeast initiator $tRNA^{Met}$ precursor. While the biosynthesis of poly(ADP-Rib) takes place by the polymerisation of nicotinamide-adenine-dinucleotide (NAD^+) leading to α(1"→2') $ribose_2$-$ribose_1$ glycosidic linkages with liberation of nicotinamide molecules (3−7), the biosynthesis pathway of Ar(p) has to be different because of the synthesis of a β(1"→2') linkage. The hypothesis of a β(1"→2') ribosylation by chemical attack of the adenosine by 5-phosphoribosyl-1-pyrophosphate (PRPP) can be advanced. Biochemical investigations have recently been undertaken in order to confirm or invalidate this hypothesis.

Regarding the real meaning of this modification, it is notable that the yeast initiator $tRNA^{Met}$ is the first tRNA characterized to date as carrying such a modified nucleotide in the T-stem at the position 64. However, six other cytoplasmic eukaryotic initiator $tRNAs^{Met}$ from plants and yeasts carry at this position 64, either a tentitatively identified Gm in *Phaseolus vulgaris* (15), or an unknown modified G in *Scenedesmus obliquus* (16), *Neurospora crassa* (17), *Torulopsis utilis* (18), *Wheat germ* (19), and *Lupinus vulgaris* (20).

Another characteristic of the yeast initiator tRNA previously described (1,21) is the fact that both the first (position 54) and the last (position 60) nucleotides of the T-loop are purines (adenosines) rather than pyrimidines (thymidines, uridines, or cytidines). This characteristic is also observed in the initiator $tRNAs^{Met}$ mentioned above, as well as in all other eukaryotic initiator $tRNAs^{Met}$ so far sequenced.

In the cytoplasmic protein synthesizing systems of eukaryotes, good indications and supporting experiments suggest that elongation factor (EF-Tu) and initiation factors (IFs) interact with the aminoacyl domain of the tRNAs consisting of the stacked aminoacyl-stem, extended to the T-stem and T-loop (22). In eukaryotic initiator $tRNAs^{Met}$, an efficient stacking could result from the nature of the nucleotide in position 64, in addition to the presence of adenosines in positions 54 and 60 mentioned above. This presence of two adenosines could be sufficient to assume an efficient stacking when the nucleotide 64 is a pyrimidine, but not when it is a purine like in plants and yeasts. In this latter case, a special modification of the purine 64 could therefore be necessary. Thus, the phosphorylated O-ribosyl-adenosine in position 64 of yeast initiator $tRNA^{Met}$ could have the specific property of allowing this tRNA to initiate protein synthesis.

Recent results obtained by others (23) support the fact that Ar(p) functions as a discriminator for elongation-initiation process. Is this the only function of Ar(p) ? Additional studies are needed to confirm this unique specific role of Ar(p) in Saccharomyces yeast. In addition, do the unknown modified Gs described above in some other eukaryotic species of plants and yeasts have the same nucleoside modification, and therefore the same function as Ar(p) ? Investigations to elucidate the chemical structure of these unknown Gs and their function are in progress in our laboratories.

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Abbreviations: initiator $tRNA^{Met}$=methionine initiator tRNA; Ar(p)=phosphorylated O-ribosyl-adenosine; Ar=phosphatase treated Ar(p)=O-ribosyl-adenosine; pAr(p)=Ar(p)-5′-phosphate; pAr(p)pG=dinucleotide-5′-terminal-phosphate containing Ar(p); Ar(p)pGp=dinucleotide-3′-terminal-phosphate containing Ar(p); Ado-Rib=O-α-D-ribosyl-(1″→2′)-adenosine; poly(ADP-Rib)=poly(adenosine-diphosphate-ribose); Ado(P)-Rib(P)=O-α-D-ribosyl-(1″→2′)-adenosine-5′,5″-diphosphate; P.O.-βE.=periodate oxidation−β-elimination procedure; EI-MS=electron impact−mass spectrometry; NMR=nuclear magnetic resonance; GC=gas chromatography; HPLC=high performance liquid chromatography.

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