Volume 12 Number 20 1984 Nucleic Acids Research

The influence of mRNA primary and secondary structure on human IFN-γ gene expression in *E. coli*

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Received 31 July 1984; Revised and Accepted 2 October 1984

ABSTRACT

Parameters influencing the efficiency of expression of the human immune interferon (IFN-γ) gene in E.coli were studied by comparing a series of eight in vitro-derived gene variants. These contained all possible combinations of silent mutations in the first three codons of the mature IFN-γ polypeptide coding sequence. Expression levels varied up to 50-fold among the different constructions. Comparison of messenger RNA secondary structure models for each variant suggested that the presence of stem-loop structures blocking the translation initiation signals could drastically decrease the efficiency of IFN-γ synthesis. With variants displaying no stable mRNA secondary structure in the region, a C→U transition at position +11 after the AUG resulted in a 5-fold increase in expression indicating that RNA primary structure also plays an important role in expression. In addition we demonstrate that, in this system, a spacing of 8 nucleotides between the Shine-Dalgarno region and AUG was optimal for gene expression and that the steady-state production level of IFN-γ rose exponentially with increasing rate of synthesis.

INTRODUCTION

The expression of heterologous genes in E.coli allows the production of large quantities of proteins that may be present only in trace amounts in their natural environment. To date, many such genes have been expressed making available certain products in amounts sufficient for clinical and industrial application (1). A knowledge of the parameters regulating the expression of foreign genes in E.coli and an ability to manipulate them to attain maximal levels of production are therefore important. The yield of a particular protein reflects intrinsic properties of the molecule, for example its stability within and toxicity to the E.coli cell (1-5), and also the efficiency of the bacterial signals for transcription and translation of the foreign gene.

Recent studies have highlighted the key role of translation efficiency in the determination of expression levels (6, 7, 8). The initiation of protein synthesis requires a start codon, usually AUG, preceded by a

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region (called the Shine-Dalgarno (SD) region) displaying some complementarity to sequences near the 3' end of 16S rRNA (9). The efficiency of initiation is affected greatly by variation of the spacing (10-15) and the base sequence (17-20) between the core sequence of the SD region and the AUG. Unlike other microorganisms, in _E.coli_ increased complementarity between the SD-region and the 16S rRNA does not necessarily lead to an increase in efficiency (7, 8). It has also been shown that sequences 5' to the SD-region of the MS2 coat protein gene are important for ribosome binding (20). Several groups have stressed the importance of mRNA secondary structure around the initiation codon in determining translational efficiency (6, 7, 21-23). In these studies it was proposed that sequence changes, either by _in vitro_ manipulation of the region 5' to the AUG or by _in vivo_ mutation, can alter the mRNA secondary structure across the ribosome binding domain. This could reduce the accessibility of either the AUG or SD-region and lead to decreased translation levels. The relative importance of primary and secondary structure effects is not clear.

Most of the studies to date have examined the effect of sequences 5' to the AUG. We have routinely observed vast differences in translation levels when the sequence 3' to an initiator AUG is modified. A role for downstream sequences was suggested by statistical analyses of the sequences surrounding the AUG codons of a large number of _E.coli_ and phage genes (8, 24, 25). These studies revealed a non-random distribution of the 20 nucleotides both before and after the AUG indicating a preference for particular bases at certain positions. In support of this, Taniguchi and Weissman (26) had found that a mutation changing the base immediately following the initiation codon of the phage Qβ coat cistron (AUGG to AUGA) resulted in more efficient ribosome binding. Hall et al. (22) showed that a mutation downstream from the AUG in the _lam_ B gene of _E.coli_ influenced translation by changing the mRNA secondary structure.

We report here a systematic analysis of the role of nucleotides 3' to the initiator AUG. In this study we have addressed the problem of the relative importance of primary and secondary structure on the initiation of protein synthesis. In addition, we have studied the effect of varying the spacing between the SD-region and the AUG. The analysis was performed using the gene for human IFN-γ, an antiviral agent which is secreted by antigen- or mitogen-stimulated lymphoid cells. IFN-γ probably plays an important role in immunoregulation and may have potential in antitumour therapy (27-30). Several other groups have expressed the IFN-γ gene in _E._

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coli (31-33). The bacterial expression vector used here contains the major leftward promoter of bacteriophage λ (P_L) and a synthetic ribosome binding site. We demonstrate that selective sequence modification within a foreign gene can significantly enhance its expression in E.coli.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The host strain for the P_L-containing expression vectors was E.coli TGE900 [F^- su^- ilv^- his^- bio (λ cI857 ΔBam ΔH1)]. This strain provides the temperature sensitive repressor λcI857. pTG11 is the IFN-γ cDNA clone and ptg951 is the initial IFN-γ expression plasmid which was derived from ptg920 (34).

General methods

Restriction enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, Boehringer Mannheim and Amersham. DNA polymerases, polynucleotide kinase, T4 DNA ligase and calf intestinal phosphatase were purchased from Boehringer Mannheim; S1 nuclease was from P-L Pharmacia. The use of these enzymes and protocols for bacterial transformation, DNA preparation and DNA sequence analysis have been described (34-37).

The oligonucleotides used were synthesized on a silica gel support following a previously described procedure (38).

Isolation of an IFN-γ cDNA clone

An IFN-γ cDNA clone was isolated from a pBR322 cDNA bank prepared using standard procedures from polyA(+) RNA from lymphocytes stimulated with phytohaemagglutinin (PHA) (28). The clone was identified using a [^{32}P]-labelled chemically-synthesized oligonucleotide probe whose sequence corresponded to a region of the previously published IFN-γ sequence (31).

Induction of cultures

Overnight cultures of E.coli strain TGE900 containing plasmid were grown at 28°C in L-Broth containing ampicillin at 100 μg/ml. These were diluted into M9 medium containing 0.2 % glucose and amino acid supplements and grown at 28°C to an OD_{660} of 0.3 (1.5 x 10^8 cells per ml). At this point the cultures were divided and parallel samples were grown at 28°C (non-induced) and at 37°C (induced) for 4 hours. De novo protein synthesis was analyzed by pulse labelling 200 μl of culture for 1 min with [^{35}S] methionine (Amersham; specific activity > 1,000 Ci/mmol) to a final concentration of 10 μCi/ml. Labelling was stopped by adding 1 ml cold PBS to

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*the samples which were then centrifuged for 10 min at 10,000 X g, resus-*pended in sample buffer and heated at 100°C for 5 min. Aliquots containing 50,000 cpm trichloroacetic acid insoluble radioactivity were loaded on 13% $NaDodSO_4$/polyacrylamide gels and analyzed by fluorography and autoradiography. [^{14}C]-labelled molecular weight markers were purchased from Bethesda Research Laboratories.

Interferon activity assays

Bacterial cultures grown and induced as described above were harvested by centrifugation and the cells resuspended in half the volume of the original culture in TGE buffer (25 mM Tris-HCl, pH 8 / 50 mM glucose / 10 mM EDTA). After sonication and addition of an equal volume of ethylene glycol the samples were centrifuged at 10,000 xg for 10 min. IFN-γ activity in the supernatent fractions was determined by measuring the inhibition of the cytopathic effect of vesicular stomatitis virus on WISH cells (27, 29). One IFN-γ unit is defined as that which provides 50 % protection against the virus. The IFN-γ expression clone ptg951 produced 2 x 10^9 U/l of culture (5 x 10^8 bacteria/ml). Assuming a specific activity of 10^8 U/mg (39) this corresponds to a level of production of ~ 10^6 molecules of IFN-γ/bacterial cell.

RESULTS

Description of the IFN-γ Expression Plasmid

The vector employed was a derivative of plasmid ptg920 whose construction has been described elsewhere (34). A synthetic ribosome binding site was cloned downstream from the P_L promoter in the N gene at the HpaI site, which had been previously modified by inserting a ClaI linker. A unique BglII site is situated within the spacer region between the SD-region and the initiation codon. The BglII site normally located upstream from P_L was replaced with a XhoI linker. The coding sequence of the IFN-γ mature polypeptide was placed immediately following the initiator ATG. The IFN-γ fragment stretches to a Sau3A site in the 3'noncoding region of the gene, 285 bp downstream from the translation termination codon. The overall structure of the plasmids and the nucleotide sequence of both the ribosome binding site and the beginning of the IFN-γ gene are shown in Fig. 1. This plasmid, ptg951, was used as the point of departure for a series of sequence modifications whose influence on IFN-γ expression was studied. Plasmid ptg951 itself contains three silent nucleotide changes with respect to the original IFN-γ sequence (31). These were T→C at position

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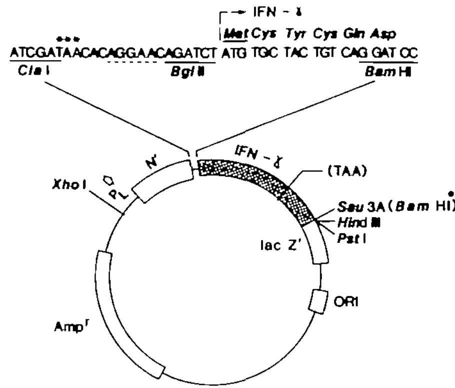


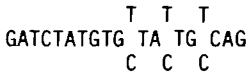
Fig. 1. Structure of the IFN-γ expression plasmid ptg951. The sequence of the ribosome binding site and the beginning of the IFN-γ gene are shown. The SD-region is marked by a dotted line and a stop codon, which is in the same reading frame as N' translation, is marked by the asterisks.

+5, C→T at position +11, and C→T at position +17 (where the A of the initiator ATG codon is position 0).

Construction of IFN-γ gene variants.

IFN-γ gene variants were obtained by replacing the BglII-BamHI fragment of ptg951, which contains the first five codons of the gene, with a series of chemically-synthesized DNA adapters.

These were prepared as two complementary populations, each containing eight oligonucleotides (20 mers). The sequence of the eight coding strands was :



Cloning of these fragments should result in the isolation of a series of plasmids which differ only in the 3rd position of the first three codons of the IFN-γ gene. These changes do not alter the original IFN-γ amino acid sequence which was presumed to start with the sequence : cys-tyr-cys (31).

The complementary oligonucleotides were phosphorylated separately using T4 polynucleotide kinase, then hybridized at 65°C for 2 h in 1 x SSC prior to ligation to ptg951 (cut with BamHI and BglII) and transformation of E.coli TGE900 cells. The DNA sequence of candidate clones was determined using the dideoxy chain termination procedure (37) directly on the plasmid DNA minipreparations (40). At least one example of each variant was obtained after sequence analysis of 36 clones. In no case was there

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Table 1 : Secondary structure models and relative IFN-γ production. The thermodynamic stability of each structure was calculated according to the method of Tinoco et al. (1973) and is expressed as a free energy (ΔG in Kcal). The percentage IFN-γ of newly-synthesized protein was obtained by densitometric tracing of the gel lanes shown in Fig. 2. IFN-γ activity was determined using the CPE inhibition assay (see Materials and Methods) and is given as a percentage of maximal expression. The initiator AUG is marked by a solid line.

No.	Most stable secondary structure around AUG	ΔG of secondary structure	% IFN-γ of newly synthesized protein	Relative IFN-γ Activity
T1	UUCAU A A GGAAC CGUA CACA A GCAU GUGU G GGACC U C AUAUA	+1.2 Kcal	24 %	20 %
T2	U A G GGAUA CACA GUCAU GUGU C C A	-1.2 Kcal	25 %	20 %
T3 pTG951	U A A G CGUA CACA GCAU GUGU CU C A	+1.2 Kcal	36 %	100 %
T4	U A G CGAUA CACA GUCAU GUGU C C A	-1.2 Kcal	33 %	100 %
T5	U A G CGUAACACA GCAUCGUGU CU A	-4.2 Kcal	19 %	10 %
T6	U G CGAUAACACA GUCAUCGUGU C A	-6.6 Kcal	11 %	4 %
T7	U A G CGUAACACA GCAUCGUGU CU A	-4.2 Kcal	14 %	10 %
T8	U G CGAUAACACA GUCAUCGUGU C A	-6.6 Kcal	11 %	2 %

evidence of a mixed population arising from incorporation of mismatched oligonucleotide fragments.

Secondary structure models

A computer-aided analysis of the nucleotide sequence of the 8 variants allowed us to construct for each case a model of the most stable secondary structure around the initiator AUG (Table 1). The stability of each projected structure was determined according to the method of Tinoco et al. (41) and the calculated free energies (ΔG) are shown. A computer scan for inverted repeats (with possible loop size 1-600 nucleotides) from -300 to +300 bp relative to the AUG revealed in each case that the structures shown represent the only significant complementarity involving this region. Examination of Table 1 shows structures with a range of free energies from relatively stable (T6 and T8 at -6.6 Kcal) to essentially

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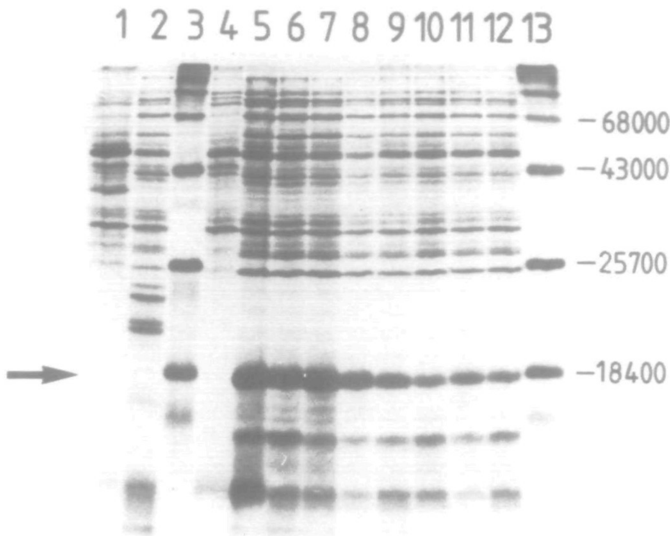

Fig. 2. Pulse-labelling analysis of extracts of E.coli producing IFN-γ. Extracts of control (vector alone) and IFN-γ plasmid-containing cultures of TGE900 were analysed. Lanes 1 and 2, non-induced and induced control; 3, M_R standards; 4, non-induced T3 (ptg951); 5-12, induced T1-T8; 13, M_R standards. The position of IFN-γ is marked by the arrow. The percentage IFN-γ in each lane was determined by densitometric tracing and is given in Table 1.

unstable (T1 and T3 at +1.2 Kcal). The stem of the structure contains a region 5' to the AGGA complementary to the first 8 nucleotides of the IFN-γ gene. The major part of the SD-region is located within the loop of this structure. Calculation of values according to an alternative method based on thermodynamic stability (42) resulted in the same conclusions (not shown).

Analysis of IFN-γ synthesis

In an attempt to determine the influence of secondary structure at the ribosome binding site on the efficiency of gene expression, the production of IFN-γ in each variant was determined in two ways. Firstly, by a pulse-labelling analysis which allowed an estimation of the level of IFN-γ synthesis as a fraction of the newly-synthesized protein and, secondly, by measuring the IFN-γ activity in crude bacterial extracts.

Induction of IFN-γ synthesis was achieved by raising the temperature

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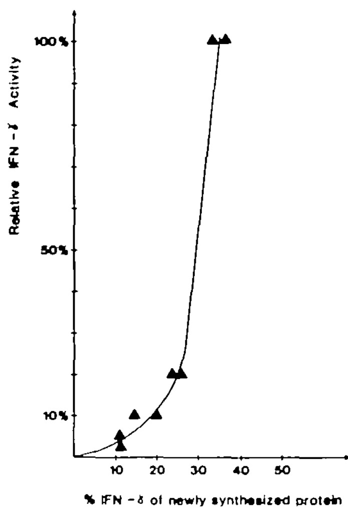

Fig. 3. Relationship between rate of synthesis and accumulation of IFN-γ. The values were taken from Table 1.

of mid-logarithmic phase cultures from 28°C to 37°C. This inactivates the thermosensitive cI857 repressor which at low temperature blocks transcription from P_L. After 4h of induction at 37°C the cultures were pulse-labelled for 1 min with [^{35}S]-labelled methionine. $NaDodSO_4$/polyacrylamide gels of cell extracts showed the specific induction of a band corresponding to IFN-γ (apparent MW 18,000) which varied in intensity among the variants (Fig. 2). Densitometric tracing (not shown) of each lane gave the percentage IFN-γ of the total protein; this ranged from 11 % (T6 and T8) to 36 % (T3) (see Table 1). Samples of post-sonication supernatents from the same cultures were tested in the IFN-γ bioassay. These results are also shown in Table 1. 100 % corresponds to the maximum expression obtained which was 2 x 10^9 IFN-γ units/litre culture. Post-sonication pellet samples contained no IFN-γ as monitored by gel analysis (not shown). It can be seen that production of IFN-γ ranged from 100 % for variants T3 and T4 to 2 % for T8. Plotting these data graphically revealed an exponential relationship between the rate of synthesis (as % newly-synthesized protein) and the accumulation of IFN-γ in the bacterial cell (Fig. 3). Measuring IFN-γ activity was the most accurate and reproducible means of estimating accumulation. Gel analysis of steady state levels was not used because of the difficulty of accurately quantifying a single band in a complex stained gel pattern.

Variation of the spacing between the SD-region and the AUG

The effect of varying the sequence between the SD-region and the AUG

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Table 2 : Modification of the nucleotide sequence between the SD-region and the AUG. Variants were obtained by modification of the BglII site of the ptg951 using DNA polymerase (Klenow fragment) and S1 nuclease. The spacing corresponds to the number of nucleotides between the end of the core AGGA of the SD-region and the AUG. IFN-γ activity is given as a percentage of maximal expression.

Nucleotide sequence between SD-region and AUG	Spacing	Relative IFN-γ activity
ΔU= .... AGGAACAGACUAUG	7	15 %
ΔAU= ... AGGAACAGCUAUG	6	15 %
ΔGAU= . ... AGGAACACUAUG	5	< 10 %
ΔAUCU= . ... AGGAACAGAUG	4	~ 0 %
pTG951 [T3] = . .. AGGAACAGAUCUAUG	8	100 %
+ AU= .. . AGGAACAGAUAUCUAUG	10	100 %
+ CAU= .... AGGAACAGAUCAUCUAUG ..	11	100 %
+ GAU= ...... AGGAACAGAUGAUCUAUG ...	11	100 %

was also studied. This was done by modifying the cohesive ends produced after cleavage at the unique BglII site in ptg951 which lies in the spacer region. After digestion with BglII, samples were treated with Klenow polymerase in the presence of either one, two, three or all four of the appropriate nucleotide triphosphate precursors. Partially "filled-in" ends were then made blunt using S1 nuclease, before recircularising the plasmid with T4 DNA ligase. This treatment resulted in a wide range of variants in this region, a sample of these are depicted in Table 2. The level of IFN-γ activity produced by these clones was determined as described above. The results show that deletion between the SD-region and the AUG decreased the level of expression. A limited increase in the spacing had little effect.

DISCUSSION

As an approach to evaluating the relative importance of the influence of mRNA primary and secondary structure on the efficiency of translation in E.coli, we have measured the effect of systematic DNA sequence modifications on the expression of a cloned foreign gene. By examination of a series of eight variants obtained by modification of the first three codons of the human IFN-γ gene it was concluded that the presence of stable secondary structure in the region of ribosome binding could account for a threefold reduction in the synthesis rate of IFN-γ (T3 compared to T6 and T8). The secondary structure models show that the AUG is partially blocked by the stem structure whereas four nucleotides of the SD-region remain exposed in the loop of the hairpin. This observation suggests that

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the accessibility of the AUG rather than the SD-region is of primary importance but it is possible that a complete absence of secondary structure in the region may be necessary for maximal ribosome binding. This supports earlier proposals that efficient initiation of translation requires an accessible AUG (2, 16, 17). Our data also indicate that structures of intermediate stability result in intermediate levels of expression (T5 and T7). In these experiments the IFN-γ sequence was the second translation unit on the mRNA, the first being a truncated N protein.

Comparison of variants T5 and T1 shows that a U→C change at position +5 destabilized the hairpin and resulted in a two-fold increase in IFN-γ production. If this change was accompanied by a C→U change at position +11 (T5→T3), a five-fold increase was observed. This effect did not occur if the change at position +11 occurred independently of the change at position +5 (T5→T7). Thus, the enhancing effect of a U (or lack of C) at position +11 was not attained in the presence of a stable hairpin structure. The base substitution at position +11 affected neither the secondary structure nor the codon usage since the codons are employed with equal frequency in E.coli (43). This observation shows that the primary structure of the region 3' to the AUG can play an important role in the determination of translational efficiency but that this effect is masked if stable secondary structure exists that could block the translation signals. The importance of position +11 is supported by the statistical analyses of Scherer et al. (24) and Gold et al. (8) which noted a preponderance of U and/or a lack of C at this position. Also, it has been shown that a C→U transition at position +11 of the λcIII gene results in increased production of cIII protein (44).

Systematic alteration of the region 5' to the AUG shows that, in this case, the minimum spacing between the SD-region and the AUG required for optimal expression was 8 nucleotides. Progressive deletion in this region led to a decrease in IFN-γ production, which reached undetectable levels when the distance fell to 4 bp. Increasing the spacing from 8 to 11 nucleotides had no effect on translation : in this experiment the 6 bases following the AGGA and the 4 bases preceding the AUG were unchanged. These sequence alterations did not significantly influence secondary structure in the region. The results emphasise the importance of appropriate spacing between the SD-region and the AUG. Shepard et al. (15) performed a similar study which concluded that the ideal spacing for the expression of fibroblast and leukocyte interferons from the trp leader ribosome binding site

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was 9 nucleotides. Other studies have demonstrated the importance of the nature of the four bases immediately following the SD-region and the three bases preceding the AUG (17, 19). The results here are consistent with the findings of these studies.

Differences in the IFN-γ synthesis rate between variants were amplified in terms of the levels of accumulation of the protein as measured in the antiviral assay. Thus, a 3-fold difference in synthesis rate between T8 and T3 resulted in a 50-fold difference in activity. Fig. 3 shows that an exponential relationship exists between the two parameters. It could be envisaged that increased efficiency of ribosome binding leads to protection of the mRNA from degradation and therefore an increased steady-state mRNA concentration. Alternatively, this could reflect a progressive saturation of the proteolytic system responsible for the hydrolysis of abnormal proteins in E.coli (2). The latter hypothesis is supported by the observation that the turnover of newly-synthesized IFN-γ was greater with higher rates of synthesis (data not shown).

Several general conclusions concerning the amelioration of foreign protein production in E.coli can be drawn from this study. For efficient translation, the initiator AUG and perhaps also the SD-region must be free from secondary structure in the 5'-terminal portion of the mRNA which may block access to the ribosomes. In addition, adequate spacing must be maintained between the SD-region and the AUG. In the absence of secondary structure, the nucleotide sequence of the region after the AUG can greatly influence synthesis rates. Thus, precise modification of the gene sequence itself, designed to disrupt secondary structure and to include optimal sequence elements, may be essential in achieving efficient expression of a given foreign gene in E.coli.

Rinderknecht et al. (45) recently published data suggesting that natural IFN-γ is processed three amino acids downstream from the predicted position (31). We are currently investigating the influence of the N-terminal residues on the biological properties of the molecule.

ACKNOWLEDGEMENTS

We thank Alain Balland, Raymonde Sauerwald and Pascale Cordier for providing the synthetic oligonucleotides. We are grateful to Dr. R. Lathe and Prof. E. Falcoff for helpful discussions and to Profs. P. Chambon and P. Kourilsky for their continued interest in the work. The expert secret-

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arial assistance of Edith Chambon and Irène Batra is gratefully acknowledged.

This project is supported by Roussel-Uclaf.

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