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A New Strategy for the Synthesis of Dinucleotides Loaded with Glycosylated Amino Acids—Investigations on in vitro Non-natural Amino Acid Mutagenesis for Glycoprotein Synthesis

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The in vitro non-natural amino acid mutagenesis method provides the opportunity to introduce non-natural amino acids site-specifically into proteins. To this end, a chemically synthesised aminoacylated dinucleotide is enzymatically ligated to a truncated suppressor transfer RNA. The loaded suppressor tRNA is then used in translation reactions to read an internal stop codon. Here we report an advanced and general strategy for the synthesis of the aminoacyl dinucleotide. The protecting group pattern developed for the dinucleotide facilitates highly efficient aminoacylation, followed by one-step global deprotection. The strategy was applied to the synthesis of dinucleotides loaded with 2-acetamido-2-deoxy-glycosylated amino acids, including N- *and* O-β*-glycosides and* O- *and* C-α*-glycosides of amino acids, thus enabling the extension of in vitro non-natural amino acid mutagenesis towards the synthesis of natural glycoproteins of high biological interest. We demonstrate the incorporation of the glycosylamino acids—although with low suppression efficiency—into the human interleukin granulocyte-colony stimulating factor (hG-CSF), as verified by the ELISA technique.*

Introduction

Protein glycosylation has been recognised as an omnipresent form of post-translational protein modification in all three kingdoms of life (eubacteria, archaea and eukaryotes), with tremendous impact on protein structure, stability and function.[1] The ubiquity of its occurrence and the diverse functions of glycosylation inspire sustained research in the field of glycoscience.[2–6] This is becoming even more the case as many human diseases are found to have close relationships with the alteration of glycoprotein glycans.[7,8]

The elucidation of a glycan's structure and function from natural glycoproteins is hampered by the appearance of heterogeneous glycan mixtures, called glycoforms, and the isolation of homogeneous glycoproteins in significant quantity is extremely difficult. For this reason, many approaches to the synthesis of glycoproteins and glycoprotein mimics are under investigation.[9–11] These approaches involve glycopeptide synthesis in combination either with native chemical ligation[12–15] or with expressed protein ligation,[16,17] chemoselective addition of glycosyl residues through modified linkages,[18–21] metabolic engineering of cell surface oligosaccharides,[22–24] enzymatic synthesis,[25,26] expression of proteins in modified organisms[27–30] and combinations thereof.[31] Depending on the application, each of these methods has its own strengths, but also inherent disadvantages and limitations. In particular, the synthesis of defined glycoproteins bearing natural glycosidic linkages by the approaches described remains a challenging task.

A general biosynthetic method for the site-specific incorporation of non-natural amino acids into proteins and peptides, based on a method for selective aminoacylation of tRNAs with non-natural amino acids developed by Hecht,[32–35] has been developed independently by the groups of Schultz[36,37] and Chamberlin.[38] Termed *in vitro non-natural amino acid mutagenesis*, this method is a powerful tool for probing a protein's structure and function and has found many applications.[39–47] It is based on the expansion of the genetic code by an artificial non-natural amino acid-charged suppressor transfer RNA (su tRNA), which reads an internal stop codon (*amber* suppression) or a four-base codon (*frameshift* suppression) at the ribosomal A-site. In contrast with the approaches mentioned

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above, this method conceptually has the potential to incorporate diverse non-natural amino acids into a protein at any desired site, regardless of the protein's size or sequence. The reported incorporation efficiencies of non-natural amino acids into proteins depend on many factors,[48–53] but explicitly on hydrophobicity[37,54,55] and steric bulk[56–58] of the amino acid to be incorporated. It was found that, as a simple rule, small and hydrophobic amino acids are incorporated with higher efficiencies. An initial approach to the incorporation of glycosylamino acids by the in vitro non-natural amino acid mutagenesis method according to this rule was reported to have been unsuccessful.[59] Nevertheless, Hecht's group succeeded in incorporating *O*-β-D-glucopyranosyl serine into firefly luciferase by this approach.[60,61] We became interested in investigating this approach with regard to the incorporation of 2-acetamido-2-deoxy sugars since these are important constituents at the reducing ends of *O*-glycan and *N*-glycan core structures.[62]

The crucial step of the technique involves the construction of an acylated su $tRNA_{CUA}$ (CUA denotes the *amber* anticodon) bearing the desired non-natural amino acid. Hecht and co-workers originally developed one approach to this problem, based on the coupling of the 2′(3′)-*O*-aminoacylated dinucleotide 5′-*O*-phosphorylcytidylyl-(3′→5′)adenosine (pCpA) mediated by T4 RNA ligase.[32–34] Chamberlin[38] and Schultz[63] simplified this method by replacing the cytidine unit of pCpA with a 2′-deoxycytidine unit, resulting in the synthesis of the dinucleotide hybrid 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′→5′)adenosine (pdCpA). It was shown that 2′(3′)-*O*-aminoacylated pdCpA derivatives are still substrates of the T4 RNA ligase and can therefore be coupled with a truncated $tRNA^{(-CA)}_{CUA}$ as efficiently as pCpA.[63] Although the substitution of pCpA by pdCpA offers several advantages, such as synthetic simplicity and product stability, the published synthetic routes to pdCpA are still tedious and rather inefficient.[53,63–69] As we started our work towards the incorporation of glycosylated amino acids into a protein, we soon came to appreciate that not only is the synthesis of the dinucleotide pdCpA challenging, but furthermore, the coupling of pdCpA with the amino acid works rather poorly in the case of 2-acetamido-2-deoxyglycosylamino acids.[62,70]

We therefore report a new synthetic approach towards aminoacylated dinucleotides, which has been used for the efficient synthesis of dinucleotides loaded with 2-acetamido-2-deoxyglycosylated amino acids in high yields. In addition, results for the incorporation of these glycosylated amino acids into human granulocyte-colony stimulating factor (hG-CSF) by in vitro non-natural amino acid mutagenesis are described. G-CSF is a hematopoietic glycoprotein cytokine, released mainly by mononuclear cells and fibroblasts. It stimulates the proliferation, differentiation and activation of cells of the granulocyte lineage into functionally mature neutrophils.[71] These cells belong to the nonspecific immune system and recognise and destroy microorganisms that enter the body. Because of its role in infection and inflammation, G-CSF has attracted much interest,[72–74] and recombinant human G-CSF (lenograstim) is commercially available and in clinical use. Recombinant hG-CSF, which is produced in Chinese hamster ovary cells, is a 174 amino acid glycoprotein with an *O*-glycosylation site at threonine 133; it exists as two glycoforms differing in a Neu5Ac residue.[75,76] It has been demonstrated that the glycosylation elongates the biological activity through prevention of polymerisation and denaturation.[77,78] Remarkably, the glycans of both glycoforms can be obtained enzymatically, starting from the Tn-antigen (GalNAc-α-Ser/Thr) structure.[79,80] The incorporation of one single glycosylamino acid would thus open up the synthesis of the natural glycoforms of hG-CSF. In view of the reported difficulties involved in the synthesis of glycoproteins by the in vitro non-natural amino acid mutagenesis method,[59–61,81,82] hG-CSF seemed to be an ideal target for investigations into improvement of this method.

Here we describe the incorporation of four glycosylamino acids into hG-CSF, including the Tn-antigen structure.

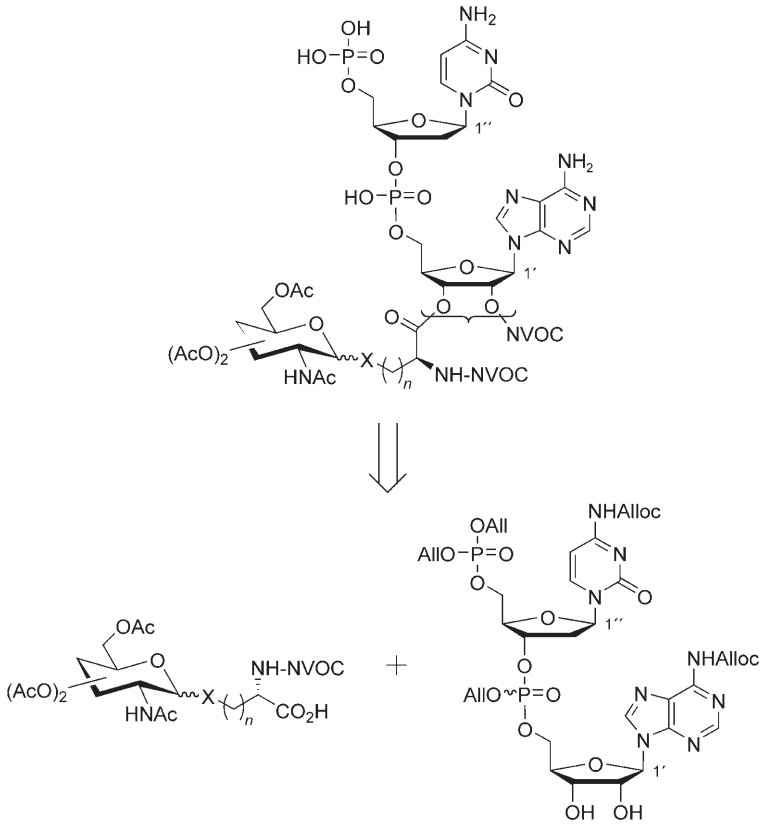
Results and Discussion

The reported routes to aminoacylated dinucleotides make use of chemoselective coupling of cyanomethyl-activated amino acids to the adenosine 2′(3′)-hydroxy group of unprotected pdCpA.[64] This approach has two main disadvantages: i) a tedious and rather inefficient synthesis of the unprotected dinucleotide is employed, and ii) comparatively large amounts of the non-natural amino acid have to be synthesised and activated as the cyanomethyl ester. Five equivalents of the activated non-natural amino acid are required to achieve satisfactory yields in the coupling reaction, rendering this approach rather unpractical for coupling with synthetically demanding non-natural amino acids. With the unprotected dinucleotide pdCpA, the use of alternative coupling reagents results in side reactions such as *N*-acylation or multiple acylation.[32,64] Recently, Hecht and co-workers published the synthesis of the β-D-gluco-, β-D-galacto-, α-D-manno- and 2-acetamido-2-deoxy-β-D-glucopyranosyl glycosides of serine and the aminoacylation of pdCpA with these glycosylamino acids by this route;[81] moderate yields for the cyanomethyl activation, high excesses of the valuable glycosylated amino acids for the coupling and only small-scale synthesis were reported.

We decided to follow a different approach (see retrosynthesis in Scheme 1), in which the aminoacylation would be performed with a fully protected dinucleotide with activation of the amino acid carboxylate, thus giving high yields of the coupling product without substantial side reactions. Crucial for this strategy is a dinucleotide protecting group pattern that can be removed under mild conditions, without the labile aminoacyl ester bond being affected. Work in this direction has been described,[36,63,66,83] but with little success with the protecting group pattern investigated. We anticipated that a dinucleotide protecting group pattern utilising allyl and allyloxycarbonyl protection for the hydroxy and amino groups, respectively, should be a promising candidate for achieving this goal.

Synthesis of the fully protected dinucleotide

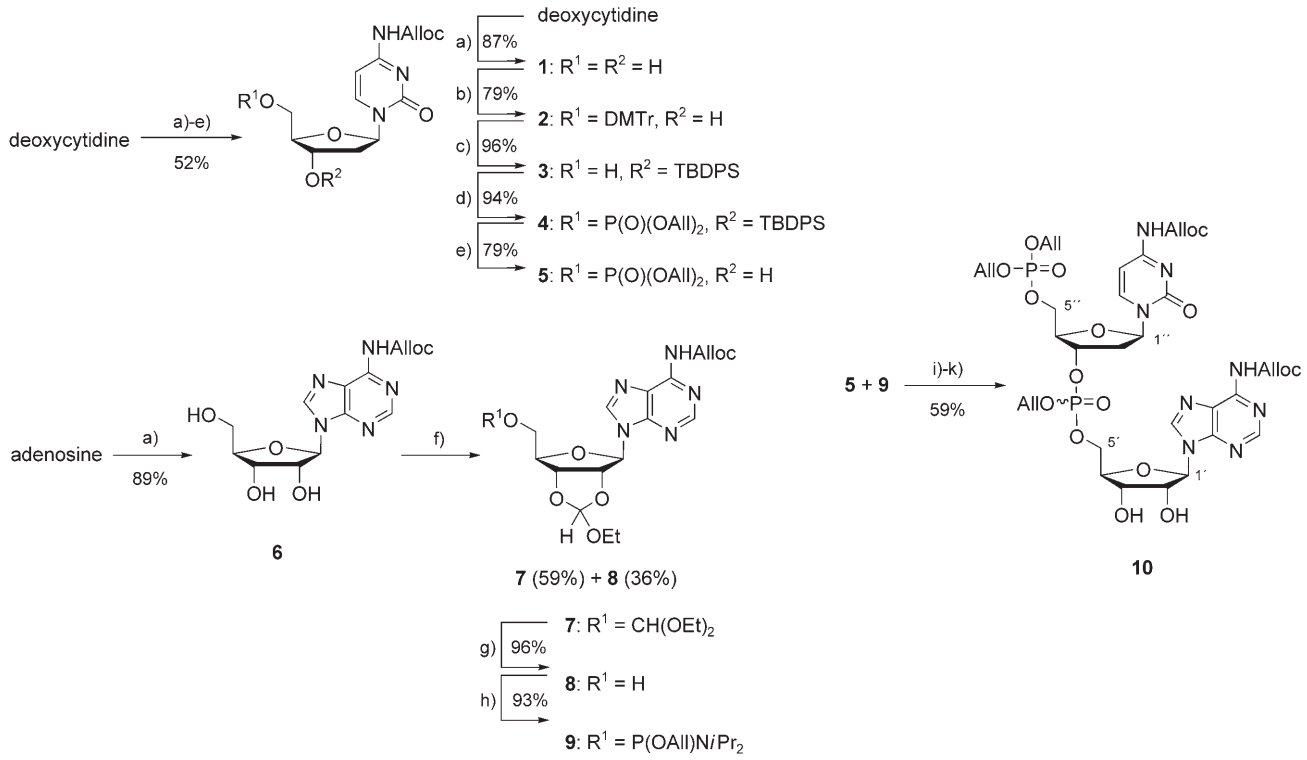
The synthesis of the fully protected dinucleotide is outlined in Scheme 2. Protection of the amino group of deoxycytidine by



Scheme 1. Retrosynthesis for the synthetic approach presented.

treatment with allyloxycarbonyl chloride under Schotten–Baumann conditions worked poorly due to the low reactivity of the allyloxycarbonyl chloride, but proceeded smoothly with *N*-methylimidazole as base, with temporary in situ TMS-protection of the hydroxy groups,[84] furnishing *N*-allyloxycarbonyl deoxycytidine **1** in 87% yield. To achieve selective 5′-phosphorylation, two protecting group manipulations could not be avoided. Firstly, selective protection of the 5′-hydroxy group with DMTr-Cl under Einhorn conditions[85] gave compound **2** (79%), and then protection of the 3′-hydroxy group with TBDPS-Cl and imidazole, followed by one-pot acidic deprotection (MeOH, TFA) of the 5′-position, gave compound **3** in excellent yield (96%). To achieve phosphorylation, bis(allyloxy)(diisopropylamino)phosphane[86] was treated with **3** with tetrazole catalysis. The resulting phosphite was oxidised to the phosphate **4** without isolation by treatment with *t*BuOOH (94%) and the TBDPS group was then removed with TBAF to afford compound **5** (79%).

The amino group of adenosine was protected by the same method[84] as for deoxycytidine, resulting in *N*-allyloxycarbonyl adenosine (**6**, 89%). Protection of the vicinal 2′,3′-diol as an orthoester by treatment with ethyl orthoformate and TCA in DMF resulted in a mixture of mono- and bis-orthoesters, but the bis-orthoester **7** could be selectively converted into the



Scheme 2. Synthesis of dinucleotide building block **10**. Reagents and conditions: a) i) HMDS, dioxane, cat. $(NH_4)_2SO_4$. ii) Alloc-Cl, *N*-methylimidazole. iii) NEt_3, MeOH. b) DMTr-Cl, pyridine. c) i) TBDPS-Cl, pyridine. ii) MeOH, TFA. d) i) $(Allyl\text{-}O)_2P\text{-}NiPr_2$, tetrazole. ii) *t*BuOOH. e) TBAF, THF. f) $HC(OEt)_3$, TCA, DMF. g) acetone, 24 h. h) $AllO\text{-}P(NiPr_2)_2$, $NHiPr_2$·tetrazole. i) tetrazole. j) *t*BuOOH. k) TFA.

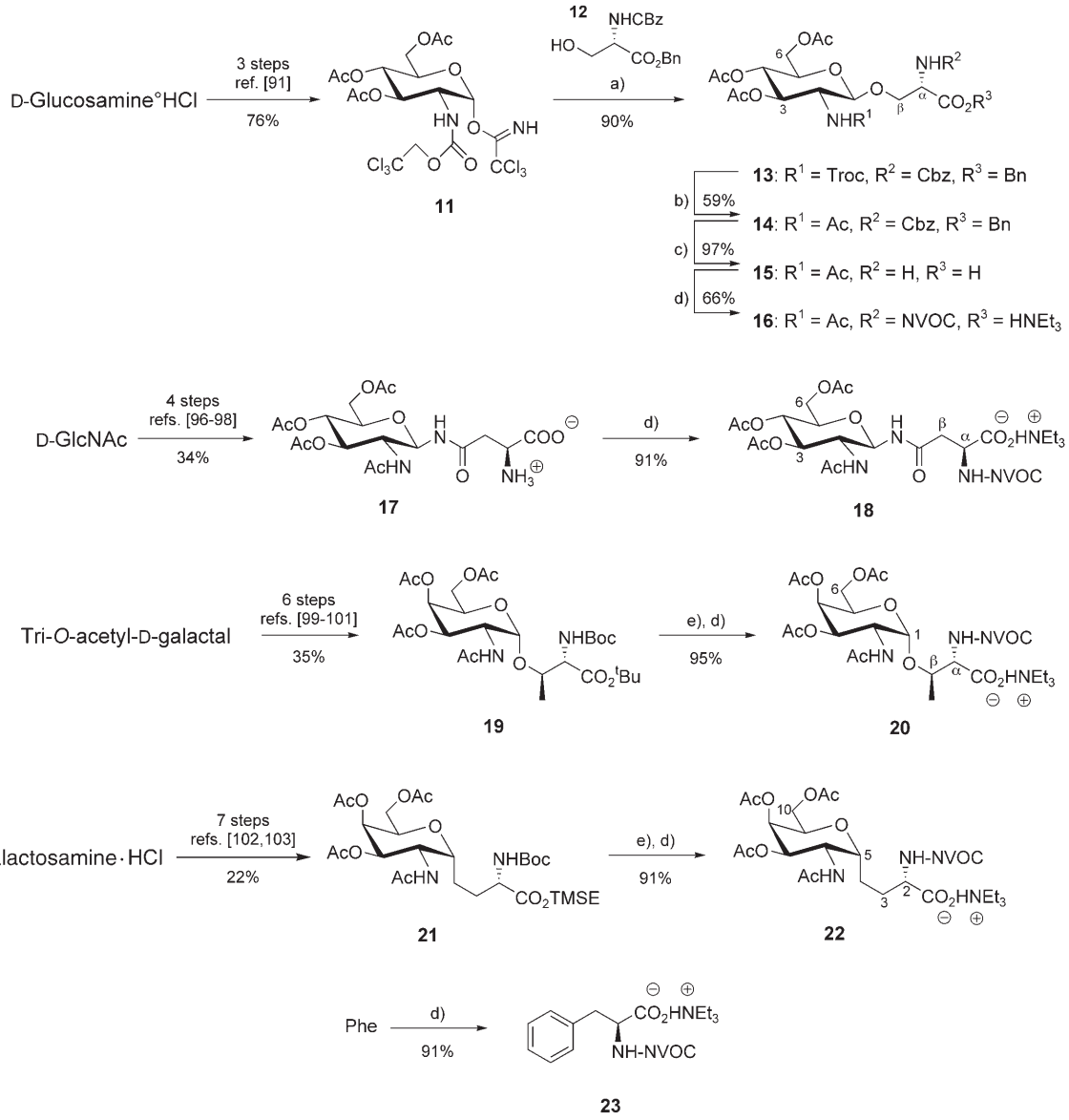
monoorthoester **8** merely by stirring in aqueous acetone overnight. Compound **8** was isolated in 93% yield. Interestingly, compound **8** was obtained as a single isomer. No NOE signals were detected for the orthoester proton to the 1-H, 4-H protons of the ribose, as would be expected for the *exo* isomer, so we assume that the *endo* isomer was formed predominantly.

For the coupling of nucleotides **5** and **8** the phosphoramidite method was applied; to this end, **8** was treated with (allyloxy)bis(diisopropylamino)phosphane to afford **9** in 93% yield, and this was immediately coupled with the deoxycytidine building block **5** with tetrazole catalysis. The next two steps, oxidation of the phosphite with *t*BuOOH and removal of the orthoester protection with TFA, were performed without purification of intermediates. Compound **10** was thus isolated in good yield (59% over three steps); partial cleavage of the phosphodiester bond probably occurred during orthoester deprotection.

In summary, the synthesis of compound **10** was performed in nine steps and in 33% overall yield. The method can be applied on a multigram scale (Scheme 2), thus permitting convenient access to this key building block.

Synthesis of glycosylated amino acids

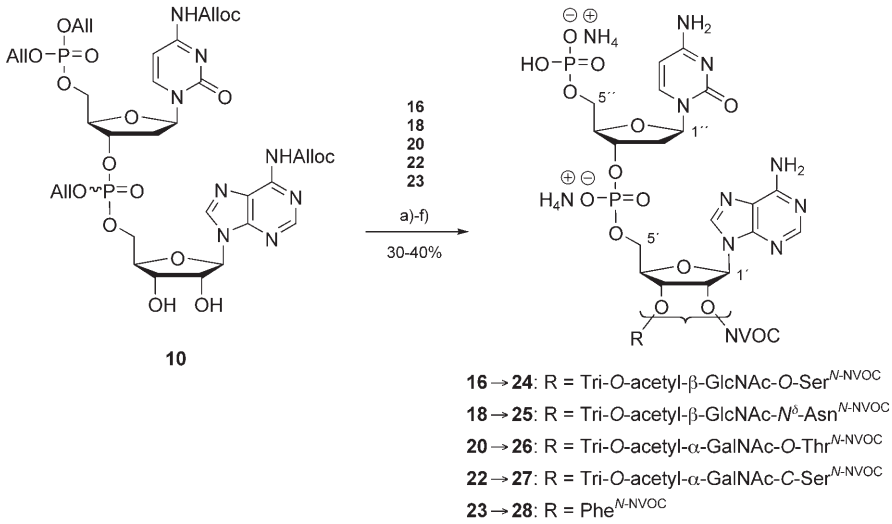
The most widespread glycosidic linkages for glycans and proteins are β-GlcNAc-*N*-Asn (*N*-glycans), β-GlcNAc-*O*-Ser/Thr and α-GalNAc-*O*-Ser/Thr (*O*-glycans), but many other, less common, glycosidic linkages have also been found.[87,88] In addition, *C*-glycosides have attracted much attention in recent years due to their metabolic stability, together with their tendency to maintain the biological activity of the *N*- and *O*-glycosidic counterparts they mimic. We therefore chose four glycosylated amino acids of high biological relevance as target structures to probe our synthetic approach. In addition to the glycosylated



Scheme 3. Syntheses of the glycosylamino acids. The references for the synthesis of the key building blocks **11**, **17**, **19** and **21** are indicated in the scheme. Reagents and conditions: a) Cat. TMS-OTf. b) Zn, Ac_2O. c) Pd/C, H_2. d) i) NVOC-Cl, $NaHCO_3$. ii) Flash chromatography. e) TFA, CH_2Cl_2.

amino acids, the phenylalanine building block[64] was also prepared. This allows for comparison with the results reported for the incorporation of phenylalanine into proteins.[36,48–50,55,60,89]

N-Protection of the amino acid provides better stability in the resulting compounds, but the protection must be compatible with the labile aminoacyl bond and with the subsequent ligation reaction. For this purpose the photocleavable nitroveratryloxycarbonyl (NVOC) group was chosen.[64] It was decided to use *O*-acetyl protection of the sugar hydroxy groups, due to simpler synthetic steps and higher corresponding yields. In addition, it was anticipated that *O*-acetyl protection should be tolerated by the ribosomal peptidyl transferase centre during peptide bond formation and, secondly, that selective cleavage after protein synthesis should be accessible. Recently, these two assumptions were reported to be correct.[16,90] The synthetic steps involved are summarised in Scheme 3.



Scheme 4. Coupling of the glycosylamino acids with the protected dinucleotide, deprotection and purification. Reagents and conditions: a) i) PyBOP, methylimidazole. ii) NVOC-Cl. b) $Pd(PPh_3)_4$, PPh_3, HCOOH/NBu_3, THF. c) RP-18 column. d) Ion-exchange column. e) HPLC, ammonium formate buffer. f) HPLC, HOAc buffer.

Tri-*O*-acetyl-β-GlcNAc-*O*-Ser$^{(N\text{-}NVOC)}$: Glycosylation of the trichloroacetimidate-activated *N*-Troc-protected glucose building block **11**[91] with commercially available *N*-benzyloxycarbonyl-L-serine benzyl ester (**12**) with TMSOTf catalysis gave the glycosylamino acid **13** in 90% yield[92,93] and, thanks to the *N*-Troc group, high β-selectivity was achieved. Exchange of the *N*-Troc for the *N*-acetyl group, by treatment with freshly activated zinc dust, proceeded in 59% yield, furnishing compound **14.**[94] Removal of the benzyl ester and *N*-benzyloxycarbonyl groups was achieved as described,[95] affording compound **15** (97%). The introduction of the NVOC group under Schotten–Baumann conditions succeeded in 66% yield to furnish compound **16**, ready for coupling with the dinucleotide.

Tri-*O*-acetyl-β-GlcNAc-*N*-Asn$^{(N\text{-}NVOC)}$: Compound **17** was synthesised as described[96–98] and the NVOC group was again introduced under Schotten–Baumann conditions to provide **18** in 98% yield.

Tri-*O*-acetyl-α-GalNAc-*O*-Thr$^{(N\text{-}NVOC)}$: Compound **19**[99–101] was transformed into the NVOC-protected glycosylamino acid under Schotten–Baumann conditions, furnishing **20** in high yield (95%).

Tri-*O*-acetyl-α-GalNAc-*C*-Ser$^{(N\text{-}NVOC)}$: The synthesis of the "*C*-glycosidic" α-GalNAc building block **21** has been described previously.[102,103] The trimethylsilylethyl (TMSE) and the Boc protecting groups were removed by treatment with TFA in dichloromethane and the NVOC group was subsequently introduced without interim purification to give building block **22** (91%).

Coupling of glycosylated amino acids and dinucleotide, deprotection and purification

The coupling procedure used for all the synthesised amino acid building blocks **16**, **18**, **20**, **22** and **23** followed a general approach that made use of PyBOP activation of the amino acid carboxylate with 1-methylimidazole catalysis (Scheme 4). Notably, no excess of the valuable glycosylated amino acid had to be applied. After coupling, the remaining 3′(2′)-hydroxy group was subsequently protected in situ as the NVOC carbonate ester to increase the stability of the aminoacyl bond. The resulting mixture of the desired compound together with minor side products such as diaminoacylated and di-NVOC-protected dinucleotides was subjected to Pd-catalysed deprotection prior to purification. The deprotection under Tsuji–Trost conditions followed a variant by Hayakawa and Nojori,[85,104] which utilises formic acid as hydride donor. Because of the complex mixture of resulting compounds—one side reaction of the Pd-catalysed *O*-allylcarbamate deprotection, for example, is formation of allylamines[105]—an effective purification procedure was established (see Experimental Section). This procedure, consisting of reversed-phase chromatography, followed by ion-exchange chromatography and two subsequent HPLC runs, yielded the desired compounds **24**–**28** in high purity on a 20–30 mg scale (30–40%). The first HPLC run serves to purify the desired compounds, while the second is performed to obtain ammonium salts, needed for the subsequent ligation to the truncated tRNA by the T4 RNA ligase. Remarkably, no β-elimination during aminoacylation with PyBOP/1-methylimidazole activation was observed; such a side-reaction was reported for coupling with cyanomethylester activation.[81]

tRNA synthesis and ligation

For the choice of the su $tRNA_{CUA}$ to be utilised, it is of importance to assure orthogonality towards the endogenous aminoacyl tRNA synthetases (ARSs) of the expression system used; otherwise deacylation of the non-natural amino acid and/or reacylation with an undesired natural amino acid can occur. The truncated tRNA species was therefore derived from yeast $tRNA^{Phe}$ and the synthesis was performed by run-off transcription from an appropriate linearised plasmid.[89] The ligation of the loaded dinucleotides and the su $tRNA^{(-CA)}_{CUA}$ was performed at 4 °C for 4 h with use of T4 RNA ligase.[106]

Cloning of the target protein hG-CSF cDNA

We used the reported hG-CSF sequence[107,108] (NCBI accession no. X03655) to design two amplification primers. These primers were used to amplify the cDNA of an RNA fraction, isolated from LPS-stimulated human whole blood, by reverse transcription PCR. The primers introduced two restriction enzyme sites, KpnI and HindIII, which were subsequently used to clone the hG-CSF cDNA into the expression vector pBluescript II KS(+), furnishing plasmid pBlue-GCSF-sigpep. After PCR-assisted subcloning (→pBlue-GCSF-wt), to obtain the hG-CSF coding sequence without the thirty amino acid signal peptide sequence, a TAG stop codon (*amber*) was introduced at position 133 by site-directed mutagenesis, providing pBlue-GCSF-T133TAG. From these expression plasmids, pBlue-GCSF-wt and pBlue-GCSF-T133TAG, the 5′-capped mRNA was obtained by in vitro transcription.

In vitro translation

The translation system, a cell lysate based on rabbit reticulocytes, was programmed with in vitro transcribed 5′-capped hG-CSF mRNA. For quantification of translation levels of hG-CSF a highly specific and sensitive sandwich ELISA was used.[109] The translation conditions were optimised with regard to Mg^{2+}, K^+, amino acid and mRNA concentrations to give the maximum yield of wild-type G-CSF (data not shown) and the determined conditions were applied to all further reactions.

The level of wild-type hG-CSF expression was $469 \pm 83\ ng\,mL^{-1}$ (Figure 1). When no mRNA was added to the cell lysate, the signal was below the detection limit of the ELISA ($<10\ pg\,mL^{-1}$ hG-CSF). To ensure the application of the ELISA for determination of stop codon suppression, it had to be demonstrated that the truncated hG-CSF, resulting from amber-133 termination, would give practically no ELISA signal. To this end, *amber*-mutated hG-CSF T133UAG mRNA alone, without any su $tRNA_{CUA}$, was added to the translation mixture. This produced ELISA signals at $0.55 \pm 0.18\ ng\,mL^{-1}$ hG-CSF (0.13% of wild-type expression), which can be explained as naturally occurring read-through of the stop codon, which is reported to take place to this extent.[110–113] Obviously, the truncated hG-CSF protein resulting from translation of the hG-CSF T133UAG mRNA is not detected by the sandwich ELISA. A factor of ≈1000 between wild-type hG-CSF and truncated hG-CSF ELISA signals ensures sensitive measurement of suppression levels by use of aminoacylated su $tRNA_{CUA}$. We define the suppression efficiency below as the ratio between hG-CSF T133UAG expression and wild-type hG-CSF expression.

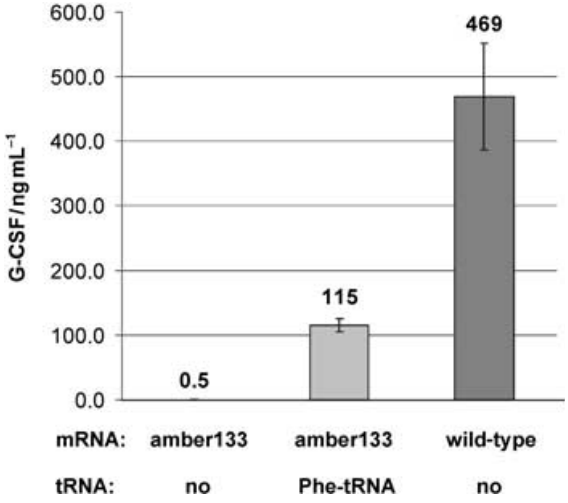


Figure 1. Expression of hG-CSF as determined by sandwich ELISA. The factor of ≈1000 between the signals for expression of the truncated hG-CSF (addition of amber133 mRNA to the cell lysate without su tRNA →0.5 ng mL^{-1} G-CSF, lane 1) and wild-type G-CSF expression (addition of wild-type mRNA to the cell lysate →469 ng mL^{-1} G-CSF, lane 3) allows sensitive measurement of suppression efficiencies by use of loaded su tRNAs. Addition of phenylalanine-loaded su tRNA results in a suppression efficiency of ≈25% (115 ng mL^{-1}, lane 2).

For reasons of comparison, we next investigated the rate of phenylalanine incorporation into hG-CSF, because substantial data for the incorporation of this amino acid into various proteins have been reported.[36,48–50,55,60,89] We determined a level of expression of $115 \pm 10\ ng\,mL^{-1}$ hG-CSF, corresponding to a suppression efficiency of $25 \pm 2\%$, which is in good agreement with reported results for phenylalanine incorporation.

The results for incorporation of the glycosylated amino acids are summarised in Figure 2, and have to be compared with the read-through level, which was $0.13 \pm 0.05\%$, as mentioned above. The suppression efficiencies for the glycosylated amino acids were $0.48 \pm 0.02\%$ for β-GlcNAc-O-Ser, $0.82 \pm 0.05\%$ for β-GlcNAc-N-Asn, $0.57 \pm 0.19\%$ for α-GalNAc-O-Thr and $0.67 \pm 0.06\%$ for α-GalNAc-C-Ser. To exclude the possibility of deacylation of the employed aminoacyl su $tRNA_{CUA}$, in combination with reacylation with a natural amino acid by an endogenous ARS, we also measured expression levels with addition of uncharged su $tRNA_{CUA}$. This level was determined to be $0.23 \pm 0.03\%$ suppression, indicating that amino acylation of su $tRNA_{CUA}$ takes place only to a very small degree. Consequently, the rates for incorporation of glycosylamino acids are significantly above read-through and reacylation levels, and thereby confirm the partial incorporation.

The results thus demonstrate that different glycosylated amino acids are incorporated, although with low suppression efficiency, into hG-CSF. The suppression efficiency might be reduced by an interfering neighbouring effect of the 2-acetamido group of the investigated sugars, resulting in faster de-

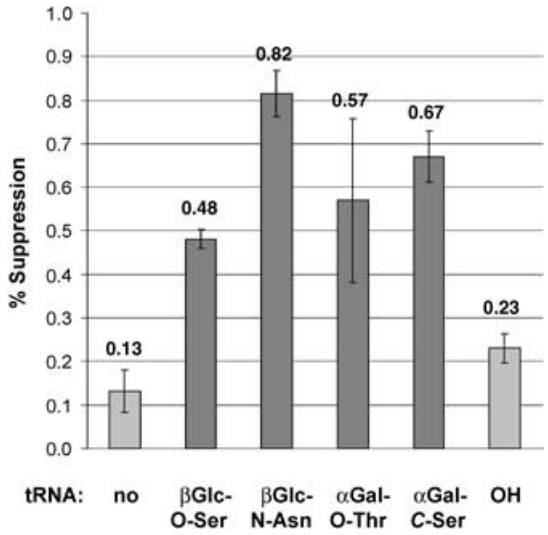


Figure 2. Suppression efficiencies for the incorporation of glycosylated amino acids into hG-CSF at position 133. The hG-CSF concentration is determined by sandwich ELISA. Calculation of suppression efficiency is done by dividing the determined hG-CSF concentration by the value of wild-type hG-CSF expression. For each experiment six data points were measured. The values for incorporation of the glycosylated amino acids are statistically significant above the negative control (OH = addition of uncharged su $tRNA_{CUA}$). $P < 0.001$ for β-GlcNAc-*O*-Ser, β-GlcNAc-*N*-Asn and α-GalNAc-*C*-Ser, and $P < 0.01$ for α-GalNAc-*O*-Thr).

acylation of the tRNA and necessitating a high excess of the su tRNA.

In addition, low degrees of incorporation of non-natural amino acids have hitherto been mainly explained by arguments involving the steric bulk of the amino acid side chain with regard to the conformational space in the A-site of the ribosome. Only recently, however, the incorporation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-glucopyranosyl-β-*O*-serine and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-galactopyranosyl-α-*O*-threonine into myoglobin by a different approach—namely, the in vivo non-natural amino acid mutagenesis method[90, 114, 115]—was reported, showing that the available space at the ribosomal A-site allows the incorporation of such bulky amino acids in principle. As a consequence, other arguments have to be considered: aminoacyl-tRNAs may be accommodated in the A-site by a ternary complex consisting of the aminoacyl-tRNA, an elongation factor (named EF-Tu in prokaryotes and EF1α in eukaryotes) and GTP. The stability of this ternary complex shows a strong influence on the accommodation process and it has been shown that misacylation of tRNAs results in a much wider range of affinities towards EF-Tu than observed with the corresponding correctly acylated tRNAs.[116–118] This supports the hypothesis that EF-Tu displays considerable specificity for both the amino acid side chain and the tRNA body, finally contributing to translation accuracy. From this, we conclude that our results for the incorporation of glycosylated amino acids into hG-GSF may be improvable by modification of either the su $tRNA_{CUA}$ or the elongation factor EF-Tu.

Conclusion

The described synthetic strategy for the synthesis of aminoacyl pdCpA offers some major advantages: the protected key building block pdCpA is obtained highly efficiently and on a large scale. The global allyl and alloc protection of pdCpA allows aminoacylation with PyBOP activation, thus avoiding the need for excesses of the employed non-natural amino acids and providing high yields. This feature extends the range of non-natural amino acids that can be coupled to the dinucleotide. The mild Pd-catalysed one-step deprotection is compatible with almost all chemical functionalities. The straightforward synthesis of the four glycosylamino acyl dinucleotides—tri-*O*-acetyl-β-GlcNAc-*N*-Asn-pdCpA, tri-*O*-acetyl-β-GlcNAc-*O*-Ser-pdCpA, tri-*O*-acetyl-α-GalNAc-*O*-Thr-pdCpA and tri-*O*-acetyl-α-GalNAc-*C*-Ser-pdCpA—which include *O*- and *N*-glycosidic bonds to 2-acetamido-2-deoxy sugars, demonstrates the power of the described strategy.

The synthesised building blocks were ligated to a truncated yeast su $tRNA^{(-CA)}_{CUA}$, and the glycosylamino acid-loaded su tRNA was subsequently employed in the in vitro non-natural amino acid mutagenesis method. Incorporation into the cytokine hG-CSF, although with low efficiency, was thus confirmed by a sensitive sandwich ELISA. This result is a further step towards the expansion of the in vitro non-natural amino acid mutagenesis method to the synthesis of glycoproteins, but at the same time it demonstrates that the incorporation of glycosylated amino acids by this method remains a challenging task. The chemical part of in vitro non-natural amino acid mutagenesis has thus been greatly improved, but further improvements are required for the incorporation of sugar-loaded su tRNAs into the protein biosynthesis machinery.

Experimental Section

Materials and reagents: Oligodeoxyribonucleotides were purchased from MWG Biotech (Ebersberg, Germany). *Pwo* DNA polymerase was purchased from Peqlab Biotechnologie GmbH (Erlangen, Germany). T4 RNA ligase and T7 RNA polymerase were purchased from MBI Fermentas (Vilnius, Lithuania). Yeast inorganic pyrophosphatase was purchased from Sigma. SUPERase®In RNase inhibitor was purchased from Ambion Ltd. (Huntingdon, UK). The Flexi® Rabbit Reticulocyte Lysate System was purchased from Promega (Madison, USA). Thin layer chromatography (TLC) was carried out on aluminium sheets coated with Kieselgel 60 F_{254} (Merck), with viewing by UV light and by staining with 15% aq. H_2SO_4 or Mostain and heat. Column chromatography was carried out with silica gel 60 (Merck, 40–63 μm, 230–400 mesh ASTM).

Instruments: ^1H (and ^{13}C) NMR spectra were recorded at 250 (63) or 600 (151) MHz with Bruker AC 250 and Bruker DRX 600 instruments. 1D TOCSY, 2D COSY and HMQC were used to assist the NMR signal assignments. MALDI-TOF mass spectra were recorded on a Bruker Biflex III spectrometer in positive, linear mode (if not indicated otherwise) with a delayed extraction MALDI source and a pulsed nitrogen laser (337 nm). Samples were dissolved in water at mM concentrations and were mixed with a standard solution of 2,5-dihydroxybenzoic acid (DHB, 10 mg mL^{-1} in 10% aqueous EtOH) in 1:1 (*v/v*) relative proportions; the mixture (0.5 μL) was loaded onto the target plate and dried under vacuum immediately

before acquisition. Elemental analysis were performed by the microanalytical lab at the Department of Chemistry of the University of Konstanz.

***N*⁴-(Allyloxy)carbonyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′-deoxycytidine (3)**: Compound **2** (3.10 g, 5.05 mmol) was dissolved in CH_2Cl_2 (50 mL). Imidazole (0.52 g, 7.60 mmol), TBDPS-Cl (2.10 mL, 7.60 mmol) and pyridine (0.81 mL, 10.1 mmol) were added, and the solution was stirred for 3 h at room temperature. MeOH (20 mL) and TFA (6 mL) were added, and the solution was stirred for an additional 3 h at room temperature. The mixture was neutralised by addition of $NaHCO_3$ solution (1% (*w*/*v*), 150 mL), the aqueous phase was extracted with CH_2Cl_2 (three times 50 mL), and the combined organic fractions were dried ($MgSO_4$), filtered and concentrated in vacuo. After silica gel column chromatography (toluene/acetone 3:1), **3** was obtained (2.66 g, 4.85 mmol, 96% yield): TLC (toluene/acetone 1:1): $R_f = 0.47$; $[\alpha]_D = +66$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 1.09$ (s, 9H; $C(CH_3)_3$), 2.1–2.3 (m, 1H; 2′-C*H*H′), 2.5–2.65 (m, 1H; 2′-CH*H*′), 3.24 (dd, $J = 3.0$ Hz, $J = 12.0$ Hz, 1H; 5′-CH_2), 3.66 (dd, $J = 2.5$ Hz, $J = 12.0$ Hz, 1H; 5′-CH_2), 4.03 (q, $J = 2.8$ Hz, 1H; 4′-*C*H), 4.40–4.45 (m, 1H; 3′-*C*H), 4.65–4.68 (m, 2H; CH_2–CH=CH_2), 5.26–5.40 (m, 2H; CH_2–CH=CH_2), 5.84–5.98 (m, 1H; CH_2–CH=CH_2), 6.24 (t, $J = 6.3$ Hz, 1H; 1′-*C*H), 7.17 (d, $J = 6.1$ Hz, 1H; H_{pyr}), 7.3–7.5 (m, 6H; H_{arom}), 7.55–7.70 (m, 4H; H_{arom}), 8.09 ppm (d, $J = 7.6$ Hz, 1H; H_{pyr}); MALDI-MS: *m/z* calcd for $C_{29}H_{35}N_3O_6Si$: 572.7 $[M+Na]^+$, 588.7 $[M+K]^+$; found 572.2, 588.2; elemental analysis calcd (%): C 63.37, H 6.42, N 7.64; found C 63.30, H 6.48, N 7.51.

***N*⁴-(Allyloxy)carbonyl-3′-*O*-(*tert*-butyldiphenylsilyl)-5′-*O*-diallylphosphoryl-2′-deoxycytidine (4)**: Compound **3** (2.20 g, 4.00 mmol) was dissolved in CH_2Cl_2 (20 mL). Di-*O*-allyl-*N*,*N*-diisopropylphosphoramidite[86] (1.73 g, 7.05 mmol) and tetrazole (1.00 g, 14.3 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h, after which *t*BuOOH (5.5 M, 1.1 mL, 5.5 mmol) was added and stirring was continued for another 20 min. The reaction mixture was concentrated in vacuo and the resulting residue was purified by silica gel column chromatography (toluene/acetone 3:1) to afford **4** (2.66 g, 3.75 mmol, 94%) as a white solid: TLC (toluene/acetone 4:1): $R_f = 0.28$; $[\alpha]_D = +36$ (c = 1.0, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 1.09$ (s, 9H; *t*Bu), 1.7–1.9 (m, 1H; 2′-C*H*H′), 2.6–2.75 (m, 1H; 2′-CH*H*′), 3.50–3.65 (m, 1H; 5′-CH_2), 3.90–4.05 (m, 1H; 5′-CH_2), 4.13 (t, $J = 2.7$ Hz, 1H; 4′-*C*H), 4.3–4.6 (m, 5H; 3′-CH, CH_2–CH=CH_2), 4.66–4.69 (m, 2H; N-CO-O-CH_2-CH=CH_2), 5.19–5.42 (m, 6H; CH_2–CH=CH_2), 5.75–6.05 (m, 3H; CH_2–CH=CH_2), 6.36–6.41 (m, 1H; 1′-*C*H), 7.23 (d, $J = 7.6$ Hz, 1H; H_{pyr}), 7.37–7.46 (m, 6H; H_{arom}), 7.61–7.66 (m, 4H; H_{arom}), 7.99 ppm (d, $J = 7.6$ Hz, 1H; H_{pyr}); MALDI-MS: *m/z* calcd for $C_{35}H_{44}N_3O_9PSi$: 732.8 $[M+Na]^+$, 748.8 $[M+K]^+$; found 732.9, 748.9; elemental analysis calcd (%): C 59.23, H 6.25, N 5.92; found C 59.01, H 6.42, N 5.41.

***N*⁴-(Allyloxy)carbonyl-5′-*O*-diallylphosphoryl-2′-deoxycytidine (5)**: Compound **4** (3.10 g, 4.37 mmol) was dissolved in THF (10 mL). TBAF solution (1 M, 10 mL, 10 mmol) was added and the reaction mixture was stirred at room temperature for 1 h, after which the reaction was stopped by addition of water (20 mL). The aqueous phase was extracted with CH_2Cl_2 (three times 60 mL), and the combined organic fractions were dried ($MgSO_4$), filtered and concentrated in vacuo. After silica gel column chromatography (EtOAc/MeOH 15:1), **5** was obtained (1.42 g, 3.02 mmol, 79%) as a white foam: TLC (EtOAc/MeOH 9:1): $R_f = 0.55$; $[\alpha]_D = +36$ (c = 1.0, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 2.09$–2.20 (m, 1H; 2′-C*H*H′), 2.67–2.75 (m, 1H; 2′-CH*H*′), 4.17–4.21 (m, 1H), 4.32–4.37 (m, 2H), 4.43–4.50 (m, 1H), 4.53–4.61 (m, 3H), 4.67–4.70 (m, 2H), 5.25–5.43 (m, 6H), 5.86–6.02 (m, 3H; CH_2–CH=CH_2), 6.27 (t, $J = 6.0$ Hz, 1H; 1′-C*H*), 7.26 (d, $J = 7.5$ Hz, 1H; H_{pyr}), 8.11 ppm (d, $J = 7.5$ Hz, 1H; H_{pyr}); MALDI-MS: *m/z* calcd for $C_{19}H_{26}N_3O_9P$: 494.4 $[M+Na]^+$, 510.4 $[M+K]^+$; found 494.2, 510.1; elemental analysis calcd (%): C 48.41, H 5.56, N 8.91; found C 48.35, H 5.61, N 8.74.

***N*⁶-(Allyloxy)carbonyl-5′-*O*-diethoxymethyl-2′,3′-*O*-ethoxymethylidene-adenosine (7)**: Compound **7** was obtained as a by-product in the synthesis of compound **8**: TLC (CH_2Cl_2/MeOH 9:1): $R_f = 0.85$; ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 1.19$ (t, $J = 7.3$ Hz, 6H; 5′-C-O-CH(O-CH_2-CH_3))$_2$, 1.33 (t, $J = 7.3$ Hz, 3H; O–CH_2–CH_3), 3.6 (q, $J = 7.3$ Hz, 4H; 5′-C-O-CH(O-CH_2-CH_3))$_2$), 3.74 (q, $J = 7.3$ Hz, 2H; O–CH_2–CH_3), 3.8–4.1 (m, 2H), 4.61 (d, $J = 1.3$ Hz, 1H; 1′-*C*H), 4.78 (dt, $J = 3.4$, 1.3 Hz, 2H; 2′-*C*H, 3′-*C*H), 5.05 (dd, 2H; 5′-CH_2), 5.15 (s, 1H; 5′-C–O–*C*H(OEt$_2$)), 5.2–5.5 (m, 3H), 5.9–6.1 (m, 1H; H_{allyl}), 6.07 (s, 1H; –*C*H–OEt), 6.30 (t, $J = 1.8$ Hz, 1H; 4′-*C*H), 8.08 (s, 1H; H_{purine}), 8.68 (br s, 1H; N*H*), 8.74 ppm (s, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{22}H_{31}N_5O_9$: 510.5 $[M+H]^+$, 532.5 $[M+Na]^+$; found 510.0, 532.0; elemental analysis calcd (%): C 51.86, H 6.13, N 13.75; found C 51.78, H 6.20, N 13.43.

***N*⁶-(Allyloxy)carbonyl-2′,3′-*O*-ethoxymethylidene-adenosine (8)**: Compound **6** (5.88 g, 16.7 mmol) was dissolved in DMF (30 mL). $HC(OEt)_3$ (4.2 mL, 25.0 mmol) and a catalytic amount of TCA (200 mg, 1.22 mmol) were added and the solution was stirred at room temperature overnight. The solution was diluted by addition of CH_2Cl_2 (100 mL) and washed with $NaHCO_3$ (1 M, 100 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The crude products were purified by silica gel column chromatography (toluene/acetone 1:1), resulting in a mixture of compounds **7** (5.02 g, 9.85 mmol, 59%) and **8** (2.45 g, 6.01 mmol). Compound **7** was converted into **8** by stirring in acetone (300 mL) at room temperature overnight. After further silica gel column chromatography (toluene/acetone 1:1) a second batch of compound **8** was obtained (3.85 g, 9.46 mmol, 96%). By this procedure, compound **8** was obtained in an overall yield of 93%: TLC (CH_2Cl_2/MeOH 9:1): $R_f = 0.74$; ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 1.33$ (t, $J = 7.3$ Hz, 3H; O–CH_2–CH_3), 3.74 (q, $J = 7.3$ Hz, 2H; O–CH_2–CH_3), 3.8–4.1 (m, 2H; 5-CH_2), 4.61 (d, $J = 1.3$ Hz, 1H), 4.78 (dt, $J = 3.4$ Hz, $J = 1.3$ Hz, 2H), 5.2–5.5 (m, 5H), 5.9–6.1 (m, 1H; H_{allyl}), 6.07 (s, 1H; C*H*–OEt), 6.30 (t, $J = 1.8$ Hz, 1H), 8.08 (s, 1H; H_{purine}), 8.68 (br s, 1H; N*H*), 8.74 ppm (s, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{17}H_{21}N_5O_7$: 408.4 $[M+H]^+$, 430.4 $[M+Na]^+$; found 408.7, 430.7; elemental analysis: calcd (%): C 50.12, H 5.20, N 17.19; found C 49.98, H 5.33, N 16.95.

[*N*⁶-(Allyloxy)carbonyl-2′,3′-*O*-ethoxymethylidene-adenosine-5′-yloxy]-allyloxy-diisopropylamino-phosphane (9): Compound **8** (1.80 g, 4.42 mmol) together with diisopropylammonium tetrazolide (0.38 g, 2.22 mmol) were dried under vacuum for 1 h and subsequently dissolved in CH_2Cl_2 (20 mL) under argon. *O*-Allyl-tetraisopropylphosphoroamidite (1.32 g, 5.02 mmol) was dissolved under argon in CH_2Cl_2 (20 mL) and added slowly to the reaction mixture. After stirring at room temperature for 3 h the mixture was concentrated in vacuo. After silica gel column chromatography (toluene/acetone 3:1, 1% (*v*/*v*) NEt_3) compound **9** was obtained, and was immediately converted in the next reaction: TLC (toluene/acetone 3:1): $R_f = 0.37$; MALDI-MS: *m/z* calcd for $C_{26}H_{39}N_6O_8P$: 633.6 $[M+K]^+$; found 633.3.

***N*⁴-(Allyloxy)carbonyl-5′-*O*-diallylphosphoryl-2′-deoxycytidylyl-{3′-(R/S)-[*O*ᴾ-allyl]-5′}-*N*⁶-(allyloxy)carbonyl-adenosine (10)**: Compound **5** (1.14 g, 2.42 mmol) and compound **9** (1.65 g, 2.77 mmol) were dried under vacuum for 1 h and were subsequently dissolved in CH_2Cl_2 (20 mL) under argon. Tetrazole (0.50 g, 7.14 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h, after which *t*BuOOH (5.5 M, 530 μL, 2.94 mmol) was injected and the solution was stirred for an additional 2 h. The mixture was

concentrated in vacuo, the residue was dissolved in MeOH (30 mL), and TFA (0.1 mL) was added. The reaction mixture was stirred at room temperature for 5 h and subsequently neutralised by addition of $NaHCO_3$ solution (1% (*w/v*), 20 mL). The aqueous phase was extracted five times with CH_2Cl_2 and the combined organic fractions were dried ($MgSO_4$), filtered and concentrated in vacuo. After silica gel column chromatography (EtOAc/MeOH 14:1, 1% (*v/v*) NEt_3), **10** was obtained (1.30 g, 1.41 mmol, 59%) as a white foam: TLC (EtOAc/MeOH 9:1): $R_f = 0.12$; $[\alpha]_D = +48$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]DMSO, TMS): $\delta = 2.32$ (m, 1H; 2″-C*H*H′), 2.62 (m, 1H; 2″-CH*H*′), 4.10–4.35 (m, 7H; 5′-CH_2, 5″-CH_2, 3′-C*H*, 4′-C*H*, 4″-C*H*), 4.48 (brs, 6H; H_{allyl}), 4.63 (m, 6H; H_{allyl}), 4.69 (brs, 1H; 2′-C*H*), 4.96 (m, 1H; 3″-C*H*), 5.1–5.5 (m, 9H; H_{allyl} and –O*H*), 5.65 (d, *J* = 4.6 Hz, 1H; –O*H*), 5.83–6.00 (m, 5H; –CH$_2$–C*H*=CH$_2$), 6.03 (d, *J* = 4.9 Hz, 1H; 1′-C*H*), 6.11 (q, *J* = 7.0 Hz, 1H; 1″-C*H*), 7.03 (d, *J* = 7.0 Hz, 1H; 5-CH_{pyr}), 8.05 (d, *J* = 7.0 Hz, 1H; 6-CH_{pyr}), 8.61 (s, 1H; H_{purine}), 8.63 (s, 1H; H_{purine}), 10.61 (brs, 1H; N*H*), 10.83 ppm (brs, 1H; N*H*); MALDI-MS: *m/z* calcd for $C_{36}H_{46}N_8O_{17}P_2$: 925.8 $[M+H]^+$, 947.8 $[M+Na]^+$, 963.8 $[M+K]^+$; found 925.8, 947.8, 963.7; elemental analysis: calcd (%): C 46.76, H 5.01, N 12.12; found C 46.64, H 5.10, N 11.85.

The syntheses of the glycosylamino acids are described in the Supporting Information.

General procedure for aminoacylation: The dinucleotide **10** (50 μmol), the appropriate amino acid (60 μmol) and the coupling reagent PyBOP (120 μmol) were dried at high vacuum for 1 h and were subsequently dissolved in dry CH_2Cl_2 (10 mL) under argon. The reaction was started by addition of *N*-methylimidazole (30 μmol) and the mixture was stirred at room temperature for 10 h. NVOC-Cl (100 μmol) and *N*-methylimidazole (100 mmol) were added and the reaction mixture was stirred at room temperature for a further 2 h. The reaction was stopped by addition of H_2O (20 mL) and HCOOH (200 μmol). The aqueous phase was extracted three times with CH_2Cl_2. The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The resulting yellow foam was subjected directly to deprotection.

General procedure for deprotection and purification: The aminoacylated dinucleotide (100 μmol), the catalyst $Pd(PPh_3)_4$ (40 μmol) and the stabiliser PPh_3 (160 μmol) were dried at high vacuum for 1 h. NBu_3 (700 μmol) and HCOOH (1.4 mmol) were dissolved in dry THF (5 mL). The solution was degassed with argon (5 min) and added to the dried compounds. The mixture was stirred at room temperature for 4 h, after which it was concentrated in vacuo. The residue was preliminarily purified by RP-18 flash chromatography (CH_3CN/H_2O (1:1)). Product-containing fractions were lyophilised and the resulting residue was subjected to ion-exchange chromatography (Amberlite IR-120, NH_4^+). Product-containing fractions were again lyophilised and the crude product was purified by preparative RP-HPLC-1: solvent A (CH_3CN/H_2O 98:2), solvent B (10X: 31.5 g ammonium formate, 3.5 mL formic acid, 20% (*v/v*) CH_3CN, with H_2O to 1 L). Gradient: 0–4 mL, 0% A; 4–38 mL, 60% A; 38–39 mL, 100% A, 39–45 mL, 100% A, 45–46 mL, 0% A, 46–51 mL, 0% A). Collected fractions were lyophilised and purified by further RP-HPLC-2: solvent A (CH_3CN/H_2O 98:2), solvent B (10 mM acetic acid, CH_3CN/H_2O 2:8). Gradient: 0–4 mL, 0% A, 4–30 mL, 45% A; 30–31 mL, 100% A; 31–36 mL, 100% A; 36–37 mL, 0% A; 37–42 mL, 0% A. The flow rate was 10–15 mL min^{-1}. The product fractions were pooled, lyophilised and stored at −20 °C.

5′-*O*-Phosphoryl-2′-deoxycytidylyl-(3′-5′)-{2′(3′)-*O*-[(2-nitroveratryloxy)carbonyl]-3′(2′)-*O*-[*N*-((2-nitroveratryloxy)carbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoysl)-L-serinyl]}-adenosine diammonium salt (24): Compound **24** was synthesised from **10** and **16** by the general procedure for aminoacylation, followed, without further purification, by the general procedure for deprotection. After purification by the general procedure for purification, **24** was obtained (30–40%): ^{1}H NMR (600 MHz, D_2O/CD_3CN 9:1, TMS): $\delta = 1.89$–2.02 (m, 13H; 3×OAc, NAc, 2″-C*H*H′), 2.46 (m, 1H; 2″-CH*H*′), 3.63–3.73 (m, 2H; 5-C*H*, 2-C*H*), 3.88–3.95 (4 s, 12H; 4×OCH_3), 3.98–4.30 (m, 10H; α-C*H*, β-CH_2, 6-CH_2, 5′-C*H*, 4″-C*H*, 5″-CH_2), 4.35–4.77 (m, 3H; 1-C*H*, 4′-C*H*, 3″-C*H*), 5.02–5.30 (m, 6H; 3-C*H*, 4-C*H*, 2×O–CO–CH_2C$_6$H$_2$(OMe)$_2$NO$_2$), 5.81–5.85 (m, 2H; 2′-C*H*, 3′-C*H*), 6.04 (d, *J* = 7.5 Hz, 1H; H_{pyr}), 6.11 (m, 1H; 1″-C*H*), 6.27 (m, 1H; 1′-C*H*), 7.85 (d, *J* = 7.5 Hz, 1H; H_{pyr}), 8.13 (brs, 1*H*; H_{purine}), 8.51 ppm (brs, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{56}H_{68}N_{12}O_{35}P_2$: 1051.3 $[M-2\times NVOC-H]^-$, 1290.3 $[M-NVOC-H]^-$, 1513.3 $[M-O-H]^-$, 1529.3 $[M-H]^-$; found 1051.5, 1290.4, 1513.6, 1529.7. RP-HPLC: t_R (RP-HPLC-1) = 31.1 min; t_R (RP-HPLC-2) = 30.8 min.

Diammonium salt of 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′)-{2′(3′)-*O*-[(2-nitroveratryloxy)carbonyl]-3′(2′)-*O*-[N^{α}-((2-nitroveratryloxy)carbonyl)-N^{β}-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-asparaginyl]}-adenosine (25): Compound **25** was synthesised from **10** and **18** by the general procedure for aminoacylation, followed, without further purification, by the general procedure for deprotection. After purification by the general procedure for purification, **25** was obtained (30–40%): ^{1}H NMR (600 MHz, D_2O/CD_3CN 9:1, TMS): $\delta = 1.99$–2.15 (m, 13H; 3×OAc, NAc, 2″-C*H*H′), 2.55 (m, 1H; 2″-CH*H*′), 3.92–3.97 (4×s, 12H; 4×OCH_3), 3.98–4.30 (m, 11H; β-CH_2, α-C*H*, 5-C*H*, 6-CH_2, 5′-CH_2, 4″-C*H*, 5″-CH_2), 4.35–4.77 (m, 3H; 1-C*H*, 4′-C*H*, 3″-CH), 5.03–5.30 (m, 6H; 3-C*H*, 4-C*H*, 2×O–CH_2–C$_6$H$_2$(OMe)$_2$NO$_2$), 5.85 (m, 1H; 3′-C*H*), 5.94–5.96 (brt, 1H; 2′-C*H*), 6.22–6.25 (t, *J* = 6.4 Hz, 1H; 1″-C*H*), 6.26–6.27 (d, *J* = 7.8 Hz, 1H; H_{pyr}), 6.42–6.43 (d, *J* = 6.9 Hz, 1H; 1′-C*H*), 7.00–7.04 (2 s, 2H; H_{arom}), 7.62–7.64 (2 s, 2H; H_{arom}), 8.12–8.13 (d, *J* = 7.8 Hz, 1H; H_{pyr}), 8.32 (s, 1H; H_{purine}), 8.65 ppm (s, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{57}H_{69}N_{13}O_{35}P_2$: 1078.3 $[M-2\times NVOC-H]^-$, 1317.3 $[M-NVOC-H]^-$, 1540.4 $[M-O-H]^-$, 1556.3 $[M-H]^-$; found 1078.2, 1317.0, 1540.2, 1556.2. RP-HPLC: t_R (RP-HPLC-1) = 30.2 min; t_R (RP-HPLC-2) = 29.2 min.

Diammonium salt of 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′)-{2′(3′)-*O*-[(2-nitroveratryloxy)carbonyl]-3′(2′)-*O*-[*N*-((2-nitroveratryloxy)carbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threoninyl]}-adenosine (26): Compound **26** was synthesised from **10** and **20** by the general procedure for aminoacylation, followed, without further purification, by the general procedure for deprotection. After purification by the general procedure for purification, **26** was obtained (30–40%): ^{1}H NMR (600 MHz, D_2O/CD_3CN 9:1, TMS): $\delta = 1.31$ (d, *J* = 6.3 Hz, 3H; CH_3), 1.82, 1.90, 1.96 (3×s, 9H; 3×OAc), 2.00 (m, 1H; 2″-C*H*H′), 2.07 (s, 3H; NAc), 2.40 (m, 1H; 2″-CH*H*′), 3.73 (s, 3H; OCH_3), 3.74 (s, 6H; 2×OCH_3), 3.78 (s, 3H; OCH_3), 3.92 (brs, 2H; 5″-CH_2), 4.04 (m, 2H; 6-CH_2), 4.08 (brs, 2H; 5′-CH_2), 4.22–4.31 (m, 3H; 4″-C*H*, 2-C*H*, 5-C*H*), 4.42–4.50 (m, 3H; α-C*H*, β-C*H*, 4′-C*H*), 4.76 (brs, 1H; 3″-C*H*), 5.01–5.24 (m, 7H; 2×O–CH_2C$_6$H$_2$(OMe)$_2$NO$_2$, 3-C*H*, 4-C*H*, 1-C*H*), 5.65 (m, 1H; 3′-C*H*), 5.77 (m, 1H; 2′-C*H*), 6.04 (t, *J* = 6.8 Hz, 1H; 1″-C*H*), 6.12 (d, *J* = 7.8 Hz, 1H; H_{pyr}), 6.31 (d, *J* = 6.5 Hz, 1H; 1′-C*H*), 6.79, 6.81, 7.37, 7.39 (4×s, 4H; H_{arom}), 8.02 (d, *J* = 7.8 Hz, 1H; H_{pyr}), 8.21, 8.53 ppm (2 s, 2H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{57}H_{70}N_{12}O_{35}P_2$: 1065.3 $[M-2\times NVOC-H]^-$, 1304.3 $[M-NVOC-H]^-$, 1527.4 $[M-O-H]^-$, 1543.3 $[M-H]^-$; found 1065.7, 1304.7, 1527.9, 1544.0. RP-HPLC: t_R (RP-HPLC-1) = 30.2 min; t_R (RP-HPLC-2) = 29.2 min.

Diammonium salt of 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′)-{2′(3′)-*O*-[(2-nitroveratryloxy)carbonyl]-3′(2′)-*O*-[*N*-((2-nitrovera-

tryloxy)carbonyl)-*C*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-L-serinyl]}-adenosine (27): Compound **27** was synthesised from **10** and **22** by the general procedure for aminoacylation, followed, without further purification, by the general procedure for deprotection. After purification by the general procedure for purification, **27** was obtained (30–40%): ^{1}H NMR (600 MHz, D_2O/CD_3CN 9:1, TMS): $\delta = 1.54$ (m, 2H; 4-CH_2), 1.78–2.05 (m, 15H; 3×OAc, NAc, 3-CH_2, 2″-CH*H*′), 2.37–2.39 (m, 1H; 2″-CH*H*′), 3.78–3.84 (4 s, 12H; 4×OCH_3), 3.95–4.08 (m, 8H; 5-C*H*, 9-C*H*, 10-CH_2, 5′-CH_2, 5″-CH_2), 4.20 (m, 1H; 4″-C*H*), 4.30–4.32 (m, 2H; 2-C*H*, 6-C*H*), 4.45 (m, 1H; 4′-C*H*), 4.77 (m, 1H; 3″-C*H*), 5.03–5.28 (m, 6H; 7-C*H*, 8-C*H*, 2×O–C$H_2C_6H_2(OMe)_2NO_2$), 5.70 (m, 1H; 3′-C*H*), 5.81 (m, 1H; 2′-C*H*), 6.00 (m, 1H; 1″-C*H*), 6.13 (d, $J = 7.8$ Hz, 1H; H_{pyr}), 6.26 (m, 1H; 1′-C*H*), 6.92, 7.04, 7.52, 7.56 (4×s, 4H; H_{arom}), 8.06 (d, $J =$ 7.8 Hz, 1H; H_{pyr}), 8.25 (s, 1H; H_{purine}), 8.54 ppm (s, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{57}H_{70}N_{12}O_{34}P_2$: 1049.3 $[M-2\times NVOC-H]^-$, 1288.3 $[M-NVOC-H]^-$, 1511.4 $[M-O-H]^-$, 1527.4 $[M-H]^-$; found 1049.3, 1287.8, 1511.2, 1527.3. RP-HPLC: t_R (RP-HPLC-1) = 29.5 min; t_R (RP-HPLC-2) = 28.9 min.

Diammonium salt of 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′)-{2′(3′)-*O*-[(2-nitroveratryloxy)carbonyl]-3′(2′)-*O*-[*N*-((2′-nitroveratryloxy)carbonyl)-L-phenylalaninyl]}-adenosine phosphate (28): Compound **28** was synthesised from **10** and **23**[64] by the general procedure for aminoacylation, followed, without further purification, by the general procedure for deprotection. After purification by the general procedure for purification, **28** was obtained (40%): ^{1}H NMR (600 MHz, D_2O/CD_3CN 9:1, TMS): $\delta = 1.87$ (m, 1H; 2″-C*H*H′), 2.20 (m, 1H; 2″-CH*H*′), 2.89–3.04 (2 m, 2H; CH_2–C_6H_5), 3.68–3.73 (4×s, 12H; 4×O–CH_3), 3.92 (brs, 2H; 5″-CH_2), 4.08 (brs, 2H; 5′-CH_2), 4.31 (m, 1H; 4″-C*H*), 4.50 (m, 1H; 4′-C*H*), 4.69 (m, 1H; 3″-C*H*), 4.85–5.17 (4×d, $J = 14.5$ Hz, 4H; 2×O–$CH_2C_6H_2(OMe)_2NO_2$), 5.52 (m, 1H; 3′-C*H*), 5.73 (m, 1H; 2′-C*H*), 5.93–5.94 (d, $J = 7.5$ Hz, 1H; H_{pyr}), 6.09 (brt, 1H; 1″-C*H*), 6.13 (d, $J = 4.9$ Hz, 1H; 1′-C*H*), 6.78–6.82 (2×s, 2H; H_{arom}), 7.19–7.25 (m, 5H; H_{arom}), 7.36–7.39 (2 s, 2H; H_{arom}), 7.89–7.90 (d, $J = 7.5$ Hz, 1H; H_{pyr}), 8.07–8.12 (2×s, 1H; H_{purine}), 8.45 ppm (s, 1H; H_{purine}); MALDI-MS: *m/z* calcd for $C_{48}H_{53}N_{11}O_{26}P_2$: 782.2 $[M-2\times NVOC-H]^-$, 1021.2 $[M-NVOC-H]^-$, 1244.3 $[M-O-H]^-$, 1260.3 $[M-H]^-$; found 782.4, 1021.0, 1244.0, 1259.6. RP-HPLC: t_R (RP-HPLC-1) = 29.6 min; t_R (RP-HPLC-2) = 30.6 min.

Construction of the tRNA encoding plasmid, run-off transcription, ligation and photodeprotection of the su tRNA are described in the Supporting Information

Cloning of the hG-CSF cDNA: Lipopolysaccharide from *Salmonella abortus equi* (Sigma, Deisenhofen, Germany, 10 μg) was added to heparinised human whole blood (200 μL) in RPMI 1640 (BioWhittaker, Verviers, Belgium, 800 μL) supplemented with penicillin (100 IU), streptomycin (100 μg mL^{-1}) and heparin (2.5 IU mL^{-1}; Liquemin®, Hoffmann LaRoche, Grenzach-Whylen, Germany). After incubation at 37 °C and under 5% CO_2 for 4 h, RNA was prepared by use of a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition, the RNA preparation was subjected to DNA digestion with the RNase-free DNase Set (Qiagen). For in vitro cDNA synthesis, one μg of total RNA was reverse-transcribed in the presence of dT16 oligonucleotides (GibcoBRL, Karlsruhe, Germany, 2.5 μM), $MgCl_2$ (5 mM), dNTP (1 mM each), RNase inhibitor (20 U) and murine leukemia virus reverse transcriptase (50 U) in PCR buffer. One tenth of the reverse-transcribed cDNA sample was subsequently subjected to the gene-specific PCR amplification step as template. The PCR reaction mixture contained $MgCl_2$ (2 mM), sense and antisense primers (400 nM), dNTP (200 μM each) and Taq-polymerase (0.5 U). Primers were CCG AAT TC CCC AGC CCC ACC CAG ACC as forward and GGC-TCG AG GCT ACA GGC AGG CAG GAG AAT GAA ACT as reverse primer (Interactiva). The specific amplification primers introduced *EcoR I* and *Xho I* restriction sites flanking the translated region of the hG-CSF gene. If not indicated otherwise, all reagents for RT-PCR were purchased from Perkin–Elmer Applied Biosystems (Weiterstadt, Germany). Thermal cycling was performed in a GeneAmp PCR System 2400 by standard RT-PCR procedures (Perkin–Elmer Applied Biosystems). The PCR product was purified by gel electrophoresis, subsequently subjected to double digestion with *EcoR I* and *Xho I* and again purified by gel electrophoresis. Afterwards, the digested PCR fragment was cloned into the vector pBluescript II KS(+) by use of the *EcoR I* and *Xho I* sites to give pBlue-GCSF-SigPep. The cloned hG-CSF cDNA insert includes a *N*-terminal 30 amino acid signal peptide sequence. The cloned insert was verified by sequencing (GATC Biotech AG, Konstanz, Germany). The sequencing indicated that from the two mRNA splicing variants of hG-CSF[108] the sequence identical to the commercially available recombinant hG-CSF[119] was cloned (Accession-No X03655).

Subcloning and site-directed mutagenesis are described in the Supporting Information.

In vitro transcription and translation in a rabbit reticulocyte lysate protein biosynthesising system: The 5′-m^{7}G(5′)ppp(5′)G-capped mRNAs of wt-hG-CSF and hG-CSF T133UAG were transcribed with Ase I-linearised plasmids pBlue-GCSF-wt and pBlue-GCSF-T133TAG (25 μg μL^{-1}) according to the manufacturer's instructions (T7 Cap-Scribe, Roche Diagnostics GmbH, Mannheim, Germany) and were stored in 10 μL aliquots at −70 °C before use.

Translation reactions (50 μL): An amino acid mixture (1 mM, 2 μL), SUPERaseIn™ ribonuclease inhibitor (1 μL), $Mg(OAc)_2$ (2.5 mM, 0.5 μL), KCl (2.5 M, 1.5 μL) and the appropriate mRNA (3.5 μg) in DEPC-treated water were added to nuclease-treated rabbit reticulocyte lysate (25 μL, 1.93 mM endogenous Mg^{2+} concentration). When a non-natural amino acid was incorporated, the appropriate aminoacyl-$tRNA_{CUA}$ (4.5 μg) was added to the incubation mixture (calculation based on yeast $tRNA^{Phe}$ value, 1 A_{260} unit ≈ 28 μg mL^{-1}). After incubation at 30 °C for 2 h, the obtained hG-CSF was quantified by sandwich ELISA by use of an antibody pair against human G-CSF (monoclonal mouse anti-human G-CSF antibody and biotinylated goat anti-human G-CSF antibody, R&D Systems, Minneapolis, USA) and recombinant hG-CSF (Amgen, Thousand Oaks, USA) as standard.[109]

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