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Synthesis of *N*-Acetylglucosaminyl Asparagine-Substituted Puromycin Analogues

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Abstract—As part of our project aimed to introduce specifically glycosylated amino acids into proteins, new glycosylated puromycin analogues were chemically synthesized. Introduction of a free *N*-acetylglucosaminyl asparaginyl side chain abolished the activity of puromycin completely, but when the sugar OH groups were rendered increasingly hydrophobic by acetylation or benzylation, up to 8% of the activity was recovered. The results of our preliminary inhibition tests suggest that the interaction of puromycin analogues and therefore also of glycosylated aminoacyl tRNA, with the ribosomal A site increases with hydrophobicity of the modifying protecting groups.

Introduction

Glycoproteins play a central role in the communication between cells, in the immune response, in tumour metastasis, as hormones and many other biological processes. Specific oligosaccharide sequences can act as characteristic recognition markers or they can regulate the enzymatic degradation of the protein component.[1] Until now it has not been possible to obtain homogeneously glycosylated proteins in an easy way by means of recombinant techniques. There is therefore a clear need for convenient methods to produce proteins with specific glycosylation patterns, natural as well as unnatural.

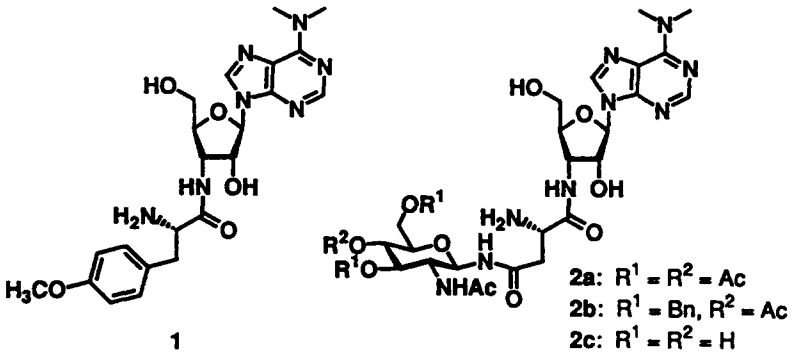
A fundamentally new approach for achieving this goal was introduced by the groups of Schultz[2] and Hecht.[3] By use of artificially aminoacylated tRNA in an *in vitro* translation system they succeeded in incorporating a number of unnatural amino acid residues into proteins. If *N*-glycosylated asparagine derivatives, or *O*-glycosylated serine or threonine derivatives were to be used as the aminoacyl components, it should thus be possible to obtain glycoproteins with defined oligosaccharide substitution patterns in a similiar way.

The synthesis of such aminoacyl tRNA derivatives is rather complex. It requires the chemical synthesis of dCA acylated at the 2'(3') hydroxy group with a glycosylated amino acid.[2,3] Subsequently, this quite labile fragment has to be coupled enzymatically to a truncated tRNA. Only after subjecting the glycosylated amino acyl tRNA to the conditions of an *in vitro* translation system will it become clear whether the method works or not. Until now, all the unnatural amino acids introduced successfully into proteins by this method are much smaller and far less complex than any glycosylated amino acid. Therefore, to avoid unnecessary synthetic efforts, it would be highly desirable to have a simple model system to test the acceptance of the sugar side chain by the ribosomal translation apparatus in advance.

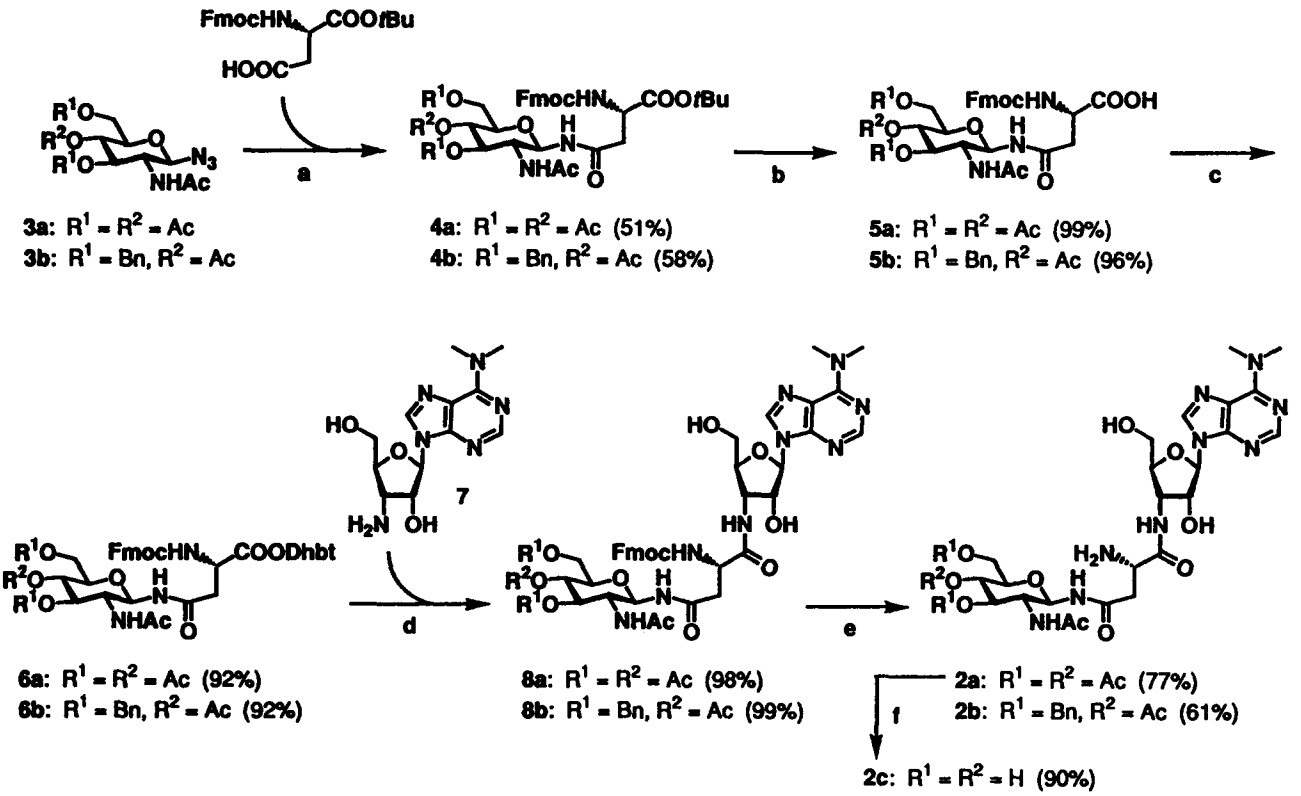
The properties of the antibiotic puromycin (**1**) might have a solution to this problem. The compound is known to compete with aminoacyl tRNA for the A site of the ribosome.[4] In the course of the translation process the oligopeptidyl tRNA bound to the P site transfers its peptidyl residue to the free amino group of the puromycin. As a result, instead of the activated 2' (3') ester of the aminoacyl tRNA, the newly formed aminoacyl puromycin contains an unreactive amide bond and the elongation of the peptide chain is terminated. The carboxy terminus of the liberated oligopeptide carries the puromycin.

A new analogue of puromycin in which the *O*-methyl tyrosinyl residue is replaced by an *N*-acetylglucosaminyl asparagine derivative might be able to inhibit protein biosynthesis in a similiar manner, so far as it is able to bind to the ribosomal A site despite its unnatural side chain. Since the amino acid moieties of puromycin and aminoacyl tRNA most likely interact with the ribosomal binding site in the same way, the inhibitory efficiency of such puromycin analogues should be a measure for the compatibility of the glycosylated amino acid with the ribosome. In addition, it should be noted that inhibition studies using puromycin analogues with unnatural aromatic amino acid residues[5] provided evidence that an increased hydrophobicity of the side chain with a proper size enhances the binding affinity.

Key words: puromycin analogues, inhibitors of protein synthesis, *N*-acetylglucosaminyl asparagine, aminoacyl tRNA, *in vitro* translation system.



Scheme 1. Puromycin (1) and the *N*-acetylglucosaminyl asparagine-substituted puromycin analogues **2a–c**.



Scheme 2. Synthesis of the puromycin analogues **2a–c**: (a) 1. H_2, Lindlar catalyst, THF; 2. EEDQ, CH_2Cl_2; (b) 98% HCOOH; (c) DhbtOH, $SOCl_2$, CH_2Cl_2; (d) DMF; (e) morpholine; (f) 1. MeOH:H_2O (4:1), K_2CO_3, pH 8.5–9.0; 2. Amberlyst 15E.

On that basis, puromycin analogues substituted by the *N*-acetylglucosaminyl asparagine **2a–c** were selected as target compounds for chemical synthesis as shown in Scheme 1. The hydrophobicity of each of the carbohydrate subunits in **2a–c** is modulated by the inclusion of three sets of hydroxyl protecting groups having different polarity. Compound **2c** is expected to be the most polar compound, whereas **2b** has the most hydrophobic side chain. Preliminary inhibition data obtained using **2a–c** in an *Eschericha coli*-derived *in vitro* transcription–translation system are also reported.

Results and Discussion

The syntheses of **2a–c** started as shown in Scheme 2, with the glycosyl azides **3a**[6] and **3b**[7] which were catalytically hydrogenated (1 bar hydrogen, Lindlar catalyst, THF). Due to their lability, the resulting amines were not isolated but immediately coupled to the β-carboxylate of Fmoc-Asp(OH)-O*t*Bu by use of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in dichloromethane to yield the *N*-acetyl glucosaminyl asparagine derivatives **4a** (51%) and **4b** (58%). Cleavage of the *tert*-butyl esters with 98% formic acid furnished the free carboxylic acids **5a** (99%) and **5b** (96%). An attempt to couple **5a** directly to the puromycin aminonucleoside (**7**) with EEDQ in dichloromethane resulted in an unsatisfactory yield of only 23%. Therefore, **5a** and **5b** were first converted to their respective dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) esters[8] **6a** and **6b** (thionyl chloride, dichloromethane, DhbtOH) in 92% yield. The reaction of these activated esters with the puromycin aminonucleoside **7** in dimethylformamide afforded the puromycin derivatives **8a** and **8b** in 98 and 99% yield, respectively. The selective removal of the Fmoc protective group was accomplished

by the treatment of **8a** and **8b** with morpholine yielding **2a** (77%) and **2b** (61%). The last step, the de-*O*-acetylation of **2a**, was complicated by two factors: the poor solubility of the starting material in organic solvents, and the high tendency for racemization at the asparagine α-carbon atom. After extensive experimentation, the deprotection was successfully accomplished by treatment with a small amount of potassium carbonate in methanol:water (4:1) at pH 8.5–9.0. After neutralization with Amberlyst 15E the completely deprotected product **2c** was obtained in 90% yield.

Selection of the 3,6-di-*O*-benzyl-4-*O*-acetyl substitution pattern in **2b** was made to allow further elaboration of the β-GlcNAc residue by glycosylation at O-4 of the precursor **3b**.

Preliminary evaluation of the inhibitory activities of **2a–c** were carried out using an *E. coli*-derived *in vitro* transcription–translation system.[5,9–11] The results of the inhibition experiments are summarized in Table 1. Puromycin showed strong inhibitory activity in the presence of DMSO, as well as without DMSO.[5,12] On the other hand, the most hydrophilic *N*-acetylglucosaminyl asparagine-substituted puromycin analogue **2c** was completely inactive. The analogues **2a** and **2b** carrying hydrophobic protective groups on the carbohydrate residue, showed only small inhibitory activity. These results indicate that the puromycin analogues **2a–c** in general are not easily acceptable at the A site of the ribosome. However, the binding affinity seems to increase with the hydrophobicity of the carbohydrate moiety. In our previous paper[5] we studied the inhibitory activity of puromycin analogues derived from non-natural aromatic amino acids to predict the steric requirements of the side chain to bind to the active centre of the ribosomal A site of *E. coli*. According to this model, the physicochemical properties of the *N*-acetylglucosamine residue rather than the asparagine residue attached to the puromycin moiety seems to be an influential factor for the ability to bind to the ribosome. Evidently, further study is needed to elucidate a more precise molecular mechanism of the inhibitory activity of puromycin analogues. In this connection, it is to be noted that Nitta *et al.* developed a more simple and sensitive *E. coli*-derived *in vitro* transcription–translation system containing a high concentration of pyridine.[13]

Table 1. Inhibitory activity[a] of puromycin (**1**) and *N*-acetylglucosaminyl asparagine substituted puromycin analogues **2a–c**

Compound	Inhibitition (%)
1 (puromycin)	83.1
2a	1.5
2b	8.9
2c	0.0

[a]Inhibition of incorporation of [^{14}C]leucine into protein using an *E. coli*-derived *in vitro* transcription–translation system was measured for one time after three repeated control experiments using puromycin. The experimental error of the measurements is estimated at 20%.

The syntheses of **2a–c** demonstrate a convenient route to glycosylated aminoacyl puromycin derivatives designed to provide an easily accessible test system for the compatibility of a carbohydrate side chain of the respective aminoacyl tRNA with the ribosomal translation apparatus. The ability of the compounds **2a** and **2b** to inhibit the protein synthesis indicates that it is probably also possible to obtain specifically glycosylated proteins from the respective glycosylated aminoacyl tRNA as outlined above. Furthermore, these compounds might be used to screen mutant cells for a higher susceptibility to the glycosylated puromycins. Such cells are expected to contain ribosomes tolerating a wider variety of glycosyl aminoacyl tRNAs.

Experimental

Chemistry

Melting points are uncorrected. Optical rotations were determined with a Perkin–Elmer Model 241 MC polarimeter. Column chromatography was performed using silica gel 60 (70–230 mesh), analytical TLC using glass plates coated with silica gel $60F_{254}$ (Merck, Darmstadt). ^{1}H NMR spectra were measured on a Jeol JNM-EX 270 instrument. The values of δ are expressed in ppm downfield from the signal of TMS. Mass spectra were taken on a Jeol JMS-HX 110 (FAB-positive; matrix: 3-nitrobenzylic alcohol + 1% CF_3COOH).

*N^4-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine tert-butyl ester (**4a**).* A solution of **3a**[5] (1.07 g, 2.87 mmol) in dry THF (25 mL) was hydrogenated in the presence of Lindlar catalyst (850 mg) for 3.5 h in the dark. After filtration over Celite, the solvent was evaporated at 40 °C under reduced pressure. After drying the residue for 3 h at 0.1 mbar, Fmoc-Asp(OH)-O*t*Bu (1.18 g, 2.87 mmol), EEDQ (1.03 g, 4.17 mmol) and 100 ml of dry CH_2Cl_2 were added and the mixture was stirred in a sealed flask for 3 days. The solution was washed with 0.5 N HCl (3 × 50 mL), saturated aqueous $NaHCO_3$ (3 × 50 mL) and once with water (50 mL). After drying over $MgSO_4$, the solvent was evaporated. The residue was sonificated for 3 min with ethanol (30 mL), then boiled for 5 min and sonificated again. After cooling the mixture to –18 °C, the product was collected by filtration and washed three times with diethyl ether. The product was dried for 18 h at 50 °C and 0.1 mbar. Yield: 1.075 g (51%) of colourless microcrystals, poorly soluble in most solvents. mp 214–215 °C, dec. $[\alpha]_D^{27}$ –1.3° (*c* 0.92; DMSO). ^{1}H NMR (270 MHz, DMSO-d_6, 300 K): 8.61 (*d*, *J* = 9.2 Hz, 1H, glc-1-NH), 7.94–7.86 (*m*, 3H, glc-2-NH, Fmoc-H), 7.70 (*d*, *J* = 7.3 Hz, 2H, Fmoc-H), 7.52 (*d*, *J* = 8.2 Hz, 1H, urethane-NH), 7.41 (*pseudo-t*, *J* = 7.1 Hz, 2H, Fmoc-H), 7.32 (*pseudo-t*, *J* = 7.3 Hz, 2H, Fmoc-H), 5.21–5.07 (*m*, 2H, glc-1,3-H), 4.82 (*pseudo-t*, *J* = 9.7 Hz, 1H, glc-4-H), 4.36–4.13 (*m*, 5H, asn-α-H, glc-6-H, Fmoc-CH_2), 3.96–3.80 (*m*, 3H, glc-2,5-H, Fmoc-9-H), 2.67–2.40 (*m*, 2H, asn-β-CH_2), 1.99, 1.96, 1.91 (three *s*, 9H, 3 × OAc), 1.72 (*s*, 3H, NAc), 1.36 (*s*, 9H, *t*Bu). FABMS: m/z 740.2 (MH^+, 77%), 684.2

(100). $C_{37}H_{45}N_3O_{13}\cdot 0.5H_2O$ (748.78), calcd: C, 59.35; H, 6.19; N, 5.61; found: C, 59.19; H, 6.06; N, 5.47.

*N^4-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine (**5a**).* A solution of **4a** (1.03 g, 1.39 mmol) in 98% HCOOH (40 mL) was stirred for 3 h and evaporated *in vacuo*. The residue was co-evaporated three times with $CHCl_3$ (40 mL) each and sonificated with diethyl ether (30 mL). The product was sucked off, washed three times with diethylether and then dried for 18 h at 50 °C and 0.1 mbar. Yield: 942 mg (99%) of colourless microcrystals, poorly soluble in most solvents; mp 210 °C, dec. $[\alpha]_D^{27}$ +1.4° (*c* 1.18; DMSO). ^{1}H NMR (270 MHz, DMSO-d_6, 300 K): 8.58 (*d*, *J* = 9.2 Hz, 1H, glc-1-NH), 7.90–7.85 (*m*, 3H, glc-2-NH, Fmoc-H), 7.70 (*d*, *J* = 7.3 Hz, 2H, Fmoc-H), 7.47–7.29 (*m*, 5H, Fmoc-H, urethane-NH), 5.21–5.06 (*m*, 2H, glc-1,3-H), 4.82 (*pseudo-t*, *J* = 9.7 Hz, 1H, glc-4-H), 4.42–4.34 (*mc*, 1H, asn-α-H), 4.30–4.16 (*m*, 4H, glc-6-H, Fmoc-CH_2), 3.96–3.79 (*m*, 3H, glc-2,5-H, Fmoc-9-H), 3.33 (*br s*, COOH, H_2O), 2.70–2.46 (*m*, 2H, asn-β-CH_2), 1.99, 1.96, 1.90 (three *s*, 9H, 3 × OAc), 1.73 (*s*, 3H, NAc). FABMS: m/z 684.2 (MH^+, 100%). $C_{33}H_{37}N_3O_{13}\cdot H_2O$ (701.68), calcd: C, 56.49; H, 5.60; N, 5.99; found: C, 56.67; H, 5.33; N, 5.89.

*N^4-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine-3-yl ester (**6a**).* A mixture of **5a** (100 mg, 0.146 mmol), DhbtOH (26 mg, 0.159 mmol), $SOCl_2$ (107 µL, 1.47 mmol) and dry CH_2Cl_2 (20 mL) was refluxed for 3 h, until the starting material was completely dissolved. After the evaporation of the solvent, the residue was sonificated with diethyl ether (30 mL). The product was sucked off and washed three times with diethyl ether and then dried for 18 h at rt and 0.1 mbar. Yield: 11.1 mg (92%) of yellowish microcrystals, mp 207–208 °C, dec. ^{1}H NMR (270 MHz, $CDCl_3$, 299 K): 8.37 (*d*, *J* = 7.6 Hz, 1H, Dhbt-H), 8.23 (*d*, *J* = 7.9 Hz, 1H, Dhbt-H), 8.01 (*pseudo-t*, *J* = 7.1 Hz, 1H, Dhbt-H), 7.86 (*pseudo-t*, *J* = 7.4 Hz, 1H, Dhbt-H), 7.74 (*d*, *J* = 7.3 Hz, 2H, Fmoc-H), 7.60 (*d*, *J* = 7.3 Hz, 2H, Fmoc-H), 7.38–7.26 (*m*, 5H, glc-1-NH, Fmoc-H), 6.47 (*d*, *J* = 9.2 Hz, 1H, urethane-NH), 6.17 (*d*, *J* = 8.2 Hz, 1H, glc-2-NH), 5.25–5.12 (*m*, 4H, asn-α-H, glc-1,3,4-H), 4.52–4.08 (*m*, 6H, glc-2,6-H, Fmoc-CH_2, Fmoc-9-H), 3.80 (*mc*, 1H, glc-5-H), 3.17–2.94 (*m*, 2H, asn-β-CH_2), 2.08 (*s*, 3H, OAc), 2.05 (*s*, 6H, 2 × OAc), 1.78 (*s*, 3H, NAc). FABMS: m/z 829.4 (MH^+, 8%), 684.3 (15). $C_{40}H_{40}N_6O_{14}\cdot H_2O$ (846.80), calcd: C, 56.74; H, 5.00; N, 9.92; found: C, 56.88; H, 4.82; N, 9.73.

*3'-(N^4-[2"-Acetamido-3",4",6"-tri-O-acetyl-2"-deoxy-β-D-glucopyranosyl]-N^2-[fluorenylmethoxycarbonyl]asparaginamido)-3'-deoxy-N,N-dimethyladenosine (**8a**).* A solution of **7** (32 mg, 0.109 mmol) and **6a** (100 mg, 0.121 mmol) in dry DMF (10 mL) was stirred for 3 h. The mixture was evaporated to dryness and the residue was first sonificated with ethanol (10 mL) for 3 min, then boiled for 5 min and sonificated again for 3 min. After cooling to –18 °C, the product was sucked off and washed three times with diethylether. It was dried for 18 h at rt and 0.1 mbar. Yield: 103 mg (98%, relative to **7**) of slightly yellowish microcrystals; mp 239 °C, dec. $[\alpha]_D^{27}$ –9.5° (*c* 0.43; DMSO). R_f 0.78 (silica gel; $CHCl_3$:MeOH 3:1 + 1% NEt_3). ^{1}H NMR (270 MHz, DMSO-d_6, 298 K): 8.59 (*d*, *J* = 9.2 Hz, 1H, glc-1-NH), 8.41 (*s*, 1H, ad-8-H), 8.20 (*s*, 1H, ad-2-H), 7.91–7.80 (*m*, 4H, Fmoc-H, rib-3-NH, glc-2-NH), 7.70 (*d*, *J* = 7.3 Hz, 2H, Fmoc-H), 7.38 (*d*, *J* = 7.2 Hz, 1H, urethane-NH), 7.34–7.28 (*m*, 4H, Fmoc-H), 6.01–5.98 (*m*, 2H, OH, rib-1-H), 5.18–5.07 (*m*, 3H, OH, glc-1,3-H), 4.83 (*pseudo-t*, *J* = 9.7 Hz, 1H, glc-4-H), 4.48–4.47 (*br m*, 3H, asn-α-H, rib-2,3-H), 4.26–4.16 (*m*, 4H, glc-6-H, Fmoc-CH_2), 3.96–3.68 (*m*, 6H, glc-2,5-H, rib-4,5-H, Fmoc-9-H), 3.61–3.31 (*br m*, 6H, NMe_2), 2.67–2.43 (*m*, 2H, asn-β-CH_2), 1.99, 1.96, 1.91 (three *s*, 9H, 3 × OAc), 1.74 (*s*, 3H, NAc). FABMS: m/z 960.4 (MH^+, 57%), 307.1 (100). FABHRMS ($C_{45}H_{54}N_9O_{15}$, MH^+): calcd 960.3739, found 960.3732.

*3'-(N^4-[2"-Acetamido-3",4",6"-tri-O-acetyl-2"-deoxy-β-D-glucopyranosyl]asparaginamido)-3'-deoxy-N,N-dimethyladenosine (**2a**).* A solution of **8a** (195 mg, 0.203 mmol) in morpholine (20 mL) was stirred under argon for 3 h. The solvent was evaporated at 40 °C bath temperature and 0.1 mbar. The residue was purified by gel filtration (Sephadex LH-20; 2 × 55 cm; $CHCl_3$:MeOH 1:1). The product fractions were pooled, evaporated to dryness, sonificated for 3 min with diethylether (50 mL), collected and washed with diethylether. Yield: 116 mg (77%) of slightly yellowish microcrystals; mp 239–240 °C, dec. $[\alpha]_D^{27}$ –17.5° (*c* 0.26; DMSO). R_f 0.27 (silica gel; $CHCl_3$:MeOH 10:1 + 1% NEt_3). ^{1}H NMR (270 MHz, DMSO-d_6, 298 K): 8.70 (*d*, *J* = 8.9 Hz, 1H, glc-1-NH), 8.41 (*s*, 1H, ad-8-H), 8.21 (*s*, 1H, ad-2-H), 8.09 (*br s*, 1H, rib-3-NH), 7.93 (*d*, *J* = 9.2 Hz, 1H, glc-2-NH), 6.09 (*br m*, 1H, OH), 6.00 (*d*, *J* = 2.6 Hz, 1H, rib-1-H), 5.21–5.08 (*m*, 3H, OH, glc-1,3-H), 4.84 (*pseudo-t*, *J* = 9.7 Hz, 1H, glc-4-H), 4.55–4.40 (*br m*, 2H, rib-2,3-H), 4.22–4.16 (*m*, 1H, rib-4-H), 4.04–3.72 (*m*, 6H, glc-2,5,6-H, rib-5-H), 3.61–3.32 (*m*, NMe_2, NH_2, asn-α-H, H_2O), 2.56–2.23 (*m*, 2H, asn-β-CH_2), 2.00, 1.97, 1.92 (three *s*, 9H, 3 × OAc), 1.77 (*s*, 3H, NAc). FABMS: m/z 738.4 (MH^+, 100%), 575.3 (15). FABHRMS ($C_{30}H_{44}N_9O_{13}$, MH^+): calcd 738.3059, found 738.3046.

*3'-(N^4-[2"-Acetamido-2"-deoxy-β-D-glucopyranosyl]-asparaginamido)-3'-deoxy-N,N-dimethyladenosine (**2c**).* To a solution of **2a** (116 mg, 0.157 mmol) in MeOH:H_2O (4:1 20 mL) a few grains (*ca* 5 mg) of K_2CO_3 were added, so that the pH was adjusted between 8.5 and 9.0. After stirring for 3 h, the mixture was neutralized by addition of Amberlyst 15E(H)$^+$, filtrated and evaporated to dryness at 40 °C bath temperature *in vacuo*. The residue was purified by gel filtration (Sephadex LH-20; 2 × 25 cm; MeOH:H_2O 4:1) in four portions. The product fractions were pooled, the MeOH was evaporated under reduced pressure at 40 °C bath temperature and the remaining water was removed by lyophilization. Yield: 86 mg (90%) of a slightly yellowish foam. $[\alpha]_D^{29}$ –13.2° (*c* 0.67; DMSO). R_f 0.26 (silica gel; $CHCl_3$:MeOH:H_2O 10:5:1 + 1% NEt_3). ^{1}H NMR (270 MHz, DMSO-d_6, 300 K): 8.41 (*s*, 1H, ad-8-H), 8.33 (*d*, *J* = 8.9 Hz, 1H, glc-1-NH), 8.21 (*s*, 1H, ad-2-H), 8.13 (*br s*, 1H, rib-3-NH), 7.82 (*d*, *J* = 8.9 Hz, 1H, glc-2-NH), 6.09 (*br mc*, 1H, OH), 6.00 (*d*, *J* = 2.6 Hz, 1H, rib-1-H), 5.16 (*br mc*, 1H, OH), 5.0–4.9 (*br m*, 2H, glc-1,3-

H), 4.84 (*pseudo-t*, J = 9.2 Hz, 1H, glc-4-H), 4.6–4.4 (*br m*, 3H, rib-2-H, 2OH), 4.05–4.03 (*m*, 1H, rib-3-H), 3.77–3.12 (*m*, glc-2,5,6-H, rib-4,5-H, asn-α-H, NMe_2, OH, NH_2, H_2O), 2.51–2.19 (*m*, 2H, asn-β-CH_2), 1.82 (*s*, 3H, NAc). FABMS: m/z 612.3 (MH^+, 11%). FABHRMS ($C_{24}H_{38}N_9O_{10}$, MH^+): calcd 612.2742, found 612.2742.

N^4-(2-Acetamido-4-O-acetyl-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine tert-*butyl ester (**4b**).* A solution of **3b**[7] (300 mg, 0.640 mmol) in dry THF (20 mL) was hydrogenated in the presence of Lindlar catalyst (250 mg) for 3.5 h in the dark. After filtration over Celite, the solution was evaporated to dryness at 40 °C bath temperature *in vacuo*. The residue was dried for 3 h at 0.1 mbar. It was treated with Fmoc-Asp(OH)-O*t*Bu (263 mg, 0.639 mmol) and EEDQ (230 mg, 0.930 mmol) in dry CH_2Cl_2 (30 mL) as described for **4a**. Yield: 308 mg (58%) of colourless microcrystals, poorly soluble in most solvents; mp 225–227 °C, dec. $[\alpha]_D^{28}$ +9.2° (*c* 0.64; DMSO). ^{1}H NMR (270 MHz, DMSO-d_6, 294 K): 8.50 (*d*, J = 9.2 Hz, 1H, glc-1-NH), 8.04 (*d*, J = 8.9 Hz, 1H, glc-2-NH), 7.87 (*d*, J = 7.3 Hz, 2H, Fmoc-H), 7.70 (*d*, J = 7.3 Hz, 2H, Fmoc-H), 7.50 (*d*, J = 8.3 Hz, 1H, urethane-NH), 7.43–7.19 (*m*, 14H, Ph-H, Fmoc-H), 5.05 (*pseudo-t*, J = 9.4 Hz, 1H, glc-1-H), 4.87 (*pseudo-t*, J = 9.4 Hz, 1H, glc-4-H), 4.59–4.17 (*m*, 8H, Ph-CH_2, Fmoc-CH_2, Fmoc-9-H, asn-α-H), 3.89–3.69 (*m*, 2H, glc-2,3-H), 3.60 (*mc*, 1H, glc-5-H), 3.50–3.33 (*m*, 2H, glc-6-H), 2.69–2.40 (*m*, 2H, asn-β-CH_2), 1.97 (*s*, 3H, OAc), 1.79 (*s*, 3H, NAc), 1.36 (*s*, 9H, *t*Bu). FABMS: m/z 836.4 (MH^+, 22%). $C_{47}H_{53}N_3O_{11}$ (835.95), calcd: C, 67.53; H, 6.39; N, 5.03; found: C, 67.42; H, 6.43; N, 4.98.

*N^4-(2-Acetamido-4-O-acetyl-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine (**5b**).* A solution of **4b** (260 mg, 0.311 mmol) was treated with 98% HCOOH (15 mL) as described for **4a**. Yield: 234 mg (96%) of colourless microcrystals, poorly soluble in most solvents; mp 213–214 °C, dec. $[\alpha]_D^{30}$ +13.4° (*c* 0.69; DMSO). ^{1}H NMR (270 MHz, DMSO-d_6, 295 K): 8.48 (*d*, J = 9.2 Hz, 1H, glc-1-NH), 8.04 (*d*, J = 8.9 Hz, 1H, glc-2-NH), 7.85 (*d*, J = 7.6 Hz, 2H, Fmoc-H), 7.64 (*d*, J = 7.3 Hz, 2H, Fmoc-H), 7.43–7.19 (*m*, 15H, urethane-NH, Ph-H, Fmoc-H), 5.07 (*pseudo-t*, J = 9.4 Hz, 1H, glc-1-H), 4.88 (*pseudo-t*, J = 9.4 Hz, 1H, glc-4-H), 4.60–4.19 (*m*, 8H, Ph-CH_2, Fmoc-CH_2, Fmoc-9-H, asn-α-H), 3.89–3.69 (*m*, 2H, glc-2,3-H), 3.50–3.34 (*m*, glc-5,6-H, COOH, H_2O), 2.71–2.50 (*m*, 2H, asn-β-CH_2), 1.91 (*s*, 3H, OAc), 1.80 (*s*, 3H, NAc). FABMS: m/z 802.3 (MNa^+, 10%), 780.3 (MH^+, 16). $C_{43}H_{45}N_3O_{11}\cdot 0.5\ H_2O$ (788.85), calcd: C, 65.47; H, 5.88; N, 5.33; found: C, 65.65; H, 5.80; N, 5.39.

*N^4-(2-Acetamido-4-O-acetyl-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl)-N^2-(fluorenylmethoxycarbonyl)asparagine 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine-3-yl ester (**6b**).* Compound **5b** (100 mg, 0.128 mmol) was treated with DhbtOH (23 mg, 0.141 mmol) and $SOCl_2$ (94 μL, 1.29 mmol) in dry CH_2Cl_2 (20 mL) as described for **6a**. Yield: 109 mg (92%) of yellowish crystals; mp 206–207 °C, dec. ^{1}H NMR (270 MHz, $CDCl_3$, 295 K): 8.32 (*d*, J = 7.9 Hz, 1H, Dhbt-H), 8.24 (*d*, J = 7.9 Hz, 1H, Dhbt-H), 8.02 (*pseudo-t*, J = 7.1 Hz, 1H, Dhbt-H), 7.86 (*pseudo-t*, J = 7.1 Hz, 1H, Dhbt-H), 7.74 (*d*, J = 7.6 Hz, 2H, Fmoc-H), 7.60 (*d*, J = 7.3 Hz, 2H, Fmoc-H), 7.40–7.26 (*m*, 15H, Ph-H, Fmoc-H, glc-1-NH), 6.45 (*d*, J = 9.2 Hz, 1H, urethane-NH), 5.35–5.21 (*m*, 4H, glc-2-NH, glc-1,4-H, asn-α-H), 4.66–4.22 (*m*, 7H, Ph-CH_2, Fmoc-CH_2, Fmoc-9-H), 3.96–3.93 (*mc*, 1H, glc-3-H), 3.78–3.49 (*m*, 4H, glc-2,5,6-H), 3.15–2.89 (*m*, 2H, asn-β-CH_2), 1.95 (*s*, 3H, OAc), 1.67 (*s*, 3H, NAc). FABMS: m/z 925.4 (MH^+, 15%), 780.3 (13). $C_{50}H_{48}N_6O_{12}\cdot H_2O$ (942.98), calcd: C, 63.69; H, 5.34; N, 8.91; found: C, 63.78; H, 5.18; N, 8.90.

*3'-(N^4-[2"-Acetamido-4"-O-acetyl-3",6"-di-O-benzyl-2"-deoxy-β-D-glucopyranosyl]-N^2-[fluorenylmethoxycarbonyl]asparaginamido)-3'-deoxy-N,N-dimethyladenosine (**8b**).* Compound **6b** (90 mg, 0.097 mmol) was treated with **7** (26 mg, 0.088 mmol) in dry DMF (10 mL) as described for **8a**. Yield: 92 mg (99%, relative to **7**) of slightly yellowish microcrystals; mp 235–236 °C, dec. $[\alpha]_D^{30}$ –8.5° (*c* 0.28; DMSO). R_f 0.45 (silica gel; $CHCl_3$:MeOH 9:1 + 1% NEt_3). ^{1}H NMR (270 MHz, DMSO-d_6, 295 K): 8.48 (*d*, J = 9.6 Hz, 1H, glc-1-NH), 8.24 (*s*, 1H, ad-8-H), 8.20 (*s*, 1H, ad-2-H), 8.06 (*d*, J = 9.2 Hz, 1H, rib-3-NH), 7.87–7.84 (*m*, 3H, glc-2-NH, Fmoc-H), 7.70 (*d*, J = 7.3 Hz, 2H, Fmoc-H), 7.50 (*d*, J = 8.3 Hz, 1H, urethane-NH), 7.43–7.19 (*m*, 14H, Ph-H, Fmoc-H), 6.03–5.99 (*m*, 2H, OH, rib-1-H), 5.19 (*br t*, J = 5.3 Hz, 1H, OH), 5.06 (*pseudo-t*, J = 9.2 Hz, 1H, glc-1-H), 4.88 (*pseudo-t*, J = 9.4 Hz, 1H, glc-4-H), 4.59–4.21 (*m*, 9H, rib-2,3-H, Ph-CH_2, Fmoc-CH_2, asn-α-H), 4.01 (*mc*, 1H, rib-4-H), 3.85–3.68 (*m*, 4H, Fmoc-9-H, glc-2,3,5-H), 3.62–3.33 (*m*, 10H, NMe_2, glc-6-H, rib-5-H), 2.65–2.49 (*m*, 2H, asn-β-CH_2), 1.91 (*s*, 3H, OAc), 1.81 (*s*, 3H, NAc). FABMS: m/z 1056.4 (MH^+, 78%). $C_{55}H_{61}N_9O_{13}\cdot 1.5H_2O$ (1083.16), calcd: C, 60.99; H, 5.96; N, 11.64; found: C, 60.91; H, 5.70; N, 11.45.

*3'-(N^4-[2"-Acetamido-4"-O-acetyl-3",6"-di-O-benzyl-2"-deoxy-β-D-glucopyranosyl]asparaginamido)-3'-deoxy-N,N-dimethyladenosine (**2b**).* **8b** (75 mg, 0.071 mmol) was treated with dry morpholine (5 mL) as described for **2a**. Yield: 36 mg (61%) of slightly yellowish microcrystals; mp 237–238 °C, dec. $[\alpha]_D^{30}$ –6.7° (*c* 0.41; DMSO). ^{1}H NMR (270 MHz, DMSO-d_6, 296 K): 8.61 (*d*, J = 8.9 Hz, 1H, glc-1-NH), 8.42 (*s*, 1H, ad-8-H), 8.21 (*s*, 1H, ad-2-H), 8.10–8.07 (*br m*, 2H, glc-2-NH, rib-3-NH), 7.36–7.19 (*m*, 10H, Ph-H), 6.09 (*br mc*, 1H, OH), 6.00 (*d*, J = 2.0 Hz, 1H, rib-1-H), 5.17 (*br mc*, 1H, OH), 5.06 (*pseudo-t*, J = 9.1 Hz, 1H, glc-1-H), 4.88 (*pseudo-t*, J = 9.4 Hz, 1H, glc-4-H), 4.61–4.37 (*m*, 6H, rib-2,3-H, Ph-CH_2), 4.05 (*mc*, 1H, rib-4-H), 3.90–3.70 (*m*, 3H, glc-2,3,5-H), 3.63–3.32 (*m*, 11H, asn-α-H, glc-6-H, rib-5-H, NMe_2), 2.56–2.28 (*m*, 2H, asn-β-CH_2), 2.01 (*br s*, 2H, NH_2), 1.91 (*s*, 3H, OAc), 1.83 (*s*, 3H, NAc). FABMS: m/z 834.3 (MH^+, 78%). $C_{40}H_{51}N_9O_{11}\cdot 1.5H_2O$ (860.92), calcd: C, 55.81; H, 6.32; N, 14.64; found: C, 55.72; H, 5.97; N, 14.50.

Inhibition experiments with puromycin analogues in an E. coli*-derived* in vitro *transcription–translation system*

For the inhibition tests, stock solutions of 12.0 mM

puromycin (1) (Sigma) and its analogues **2a–c** in DMSO were prepared. Since **2a** and **2b** were almost insoluble in water, stock solutions of all the tested puromycin analogues in DMSO were prepared, resulting in a DMSO concentration of 8.7% in the final reaction mixture. The *E. coli* S30 extract used for the cell-free protein synthesis was obtained according to Pratt[10] from *E. coli* A19 (metB, rna). T7 RNA polymerase was prepared according to Zawadzki and Gross.[14] Inhibition activity was measured in a system for the *in vitro* synthesis of T7 gene 10 protein encoded by pGEMEX-1 (Promega) under the control of the bacteriophage T7 promotor.[4] The reaction mixture of the *in vitro* transcription–translation system consisted of (per 15 μL) 55 mM HEPES-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP and UTP, 27 mM PEP, 6.0% PEG 1000, 0.64 mM 3',5'-cyclic AMP, 68 μM L(–)-5-formyl-5,6,7,8-tetrahydrofolic acid, 175 μg mL^{-1} *E. coli* tRNA, 210 mM potassium glutamate, 27.5 mM ammonium acetate, 14 mM magnesium acetate, 0.46 mM L-[^{14}C]leucine (267 MBq $mmol^{-1}$, Amersham), 0.5 mM of each of the other 19 amino acids, 6.7 μg mL^{-1} pGEMEX-1, 93 μg mL^{-1} T7 RNA polymerase, 3.6 μL S30 extract, 8.7% DMSO and 1.0 mM puromycin (1) or its analogues **2a–c**. The reaction mixture was incubated at 37 °C for 30 min. The amount of [^{14}C]leucine incorporated into the polypeptide was determined by liquid scintillation counting of the material insoluble in trichloroacetic acid. The inhibitory activity of puromycin (1) and its analogues **2a–c** was expressed relative to a control experiment without an inhibitor. The experimental error of the measurements was estimated at 20%.

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