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Shorter puromycin analog synthesis by means of an efficient Staudinger–Vilarrasa coupling

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Abstract—An efficient Staudinger–Vilarrasa coupling generates amides from azides and 1-hydroxybenzotriazole esters of amino- or hydroxy acid derivatives in very high isolated yields and purity. New puromycin analogs, mostly putative biosynthetic intermediates, were synthesized in nine steps from adenosine.

1. Introduction

1.1. Puromycin's mode of action as an antibiotic

Puromycin (**1**) is long known as an antibiotic agent produced by *Streptomyces alboniger*. This molecule is a 3′-amino-3′-deoxynucleoside derivative bearing an amide linked L-α-aminoacid. From a structural point of view, it strongly resembles the 3′-terminus of any aminoacylated transfer RNA (Fig. 1, left).

Like the natural substrate, puromycin is recognized by the ribosomal A-site, albeit independently of the codon, where the antibiotic's α-amino group accepts a nascent peptide chain from the neighboring P-site bound peptidyl transfer RNA, which leads to truncated, dysfunctional puromycyl peptides. Hence, its mode of action consists in irreversibly inhibiting the ribosomal bacterial protein synthesis. However, puromycin metabolism is accompanied by the formation of the toxic deacylated aminodeoxynucleoside, PAN.[1] Therefore, this natural antibiotic cannot be used for medical purposes for humans. Moreover, puromycin suffers somewhat from a lack of selectivity toward prokaryotic cells in the sense that both bacterial and mammalian systems recognize puromycin and in vitro binding affinity differences between prokaryotic and eukaryotic ribosomes are sometimes arguably significant.[2] In addition, bacterial resistance toward puromycin may arise.[3] Thus, the search for new puromycin analogs may serve simultaneously several aims: to find a novel broad band antibiotic agent, to better comprehend the ribosomal protein synthesis mechanism[4] and to validate a putative biosynthetic pathway of puromycin in *S. alboniger*.

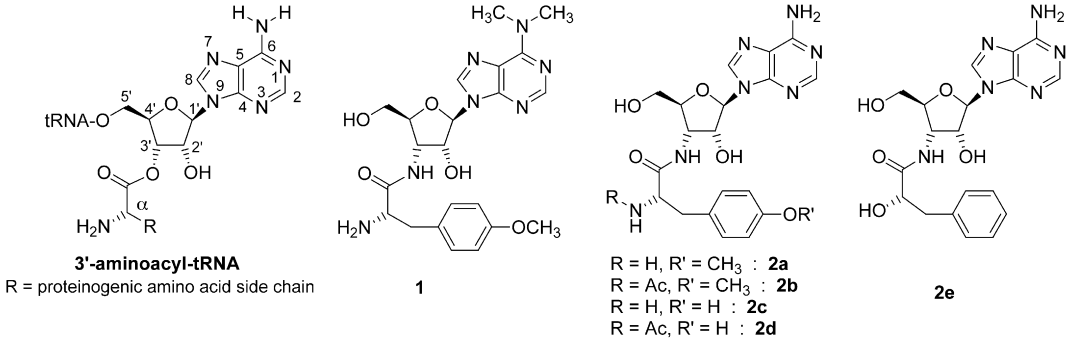

Figure 1. Natural compounds (left) and putative biosynthetic or other mimics (**2a–e**).

Keywords: Nucleosides; Amide coupling; Antibiotic; Biosynthesis.
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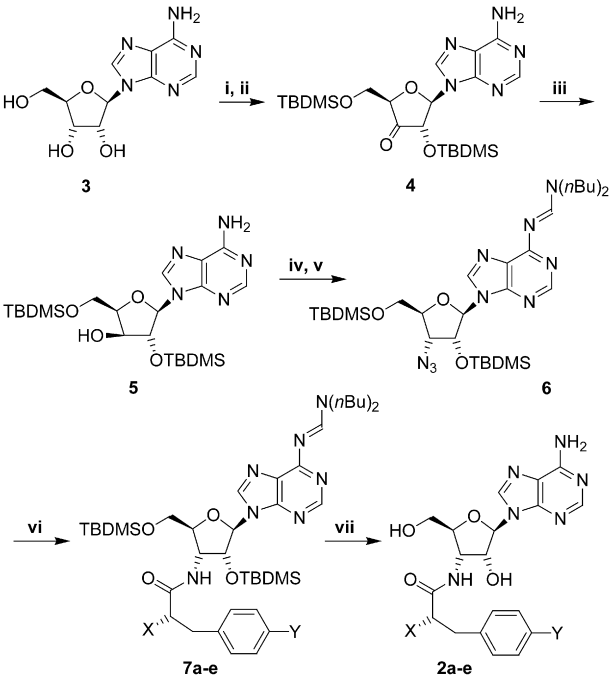
Several syntheses of a variety of puromycin analogs have already been described.[5]

1.2. Puromycin's biosynthesis and detoxification

The main aim of this work was to synthesize putative biosynthetic intermediates of puromycin that could validate a biosynthetic pathway in *S. alboniger* that was proposed by Jiménez and collegues.[6] The remaining uncertainties of the biosynthesis reside, on the one hand, on the order of the addition of three methyl groups—two onto the 6-amino group of puromycin's adenine moiety and one onto the phenol function of the attached tyrosine moiety. On the other hand, the biosynthesis of puromycin requires two enzymes that interfere with α-*N*-acetylated puromycin derivatives, in order to protect the host organism from its own metabolite. Since puromycin's α-amino group functions as the bioactive nucleophilic center in the ribosomal A-site, the inhibition or perturbation of the order of addition and hydrolysis of an *N*-acetyl group would pose severe autotoxicity problems.[3a,7] It was also shown that *S. alboniger* developed a different kind of resistance against the action of puromycin by way of the transmembrane protein Pur8, possibly by promoting an active efflux energized by a proton-dependent electrochemical gradient.[8] Actually, this is also how other bacteria, mutants of the *Klebsiella pneumoniae* strain ECL8 or *Enterobacter cloacae*,[3] manage to develop some resistance against puromycin. Moreover, *K. pneumoniae* shows an *N*-acetyl transferase activity as well,[9] which may contribute to this resistance phenomenon. Through the in-depth knowledge about these detoxification processes it should be nowadays possible to develop a rational biomedical strategy against both resistance mechanisms, in order to eventually use some puromycin analog as a new broad band antibiotic—it would be one of the suicidal kind, an irreversible inhibitor that would provoke the accumulation of truncated prokaryotic peptides, which should provoke an enhanced human immune response to additionally fight the infection.

1.3. Synthesis of puromycin analogs

To first study the proposed biosynthetic pathway of puromycin, we decided to synthesize three new puromycin analogs **2b–d** (Fig. 1) using an efficient synthetic procedure developed in our labs. We already synthesized analog **2a**,[5m] while α-hydroxy-α-deamino analog **2e** will serve a different purpose. One key-step of the synthesis is the usage of the so-called modified Staudinger reaction uncovered by Vilarrasa and collegues and developed by Vilarrasa and others,[10] an attractive water-compatible variant of which has recently become known through Bertozzi and collegues as the Staudinger ligation,[11] and that we should like to call the Staudinger–Vilarrasa coupling. It proceeds from organoazide **6** and a suitably activated *N*-protected α-aminoacid or unprotected α-hydroxy acid (Scheme 1). The formerly used reduction of the 3′-azido group to an amino group becomes obsolete. We gain one synthetic step, obtain most of time a higher coupling yield and need not care about the chemical stability of the 3′-amino derivative, which is difficult to stock owing to a slow cleavage of the base-sensitive nucleobase protecting group. Other optimized protocols also contributed to a more efficient synthesis with respect to the one we published.[5m]



7a: X = NHFmoc, Y = OCH_3
7b: X = NHAc, Y = OCH_3
7c: X = NHFmoc, Y = OH
7d: X = NHAc, Y = OH
7e: X = OH, Y = H

2a: X = NH_2, Y = OCH_3
2b: X = NHAc, Y = OCH_3
2c: X = NH_2, Y = OH
2d: X = NHAc, Y = OH
2e: X = OH, Y = H

Scheme 1. (i) TBDMSCl, Py, rt, 72 h; SiO_2, 48%; (ii) CrO_3, Py, Ac_2O, CH_2Cl_2, rt, 3 h; SiO_2, 81%; (iii) $NaHB(OAc)_3$, CH_3CN/AcOH (50:3 v/v), −18 °C, 5 h; CH_2Cl_2/$NaHCO_3$–H_2O_2 workup, 90%; (iv) $(CH_3O)_2CH$–$N(n\text{-}Bu)_2$, MeOH, rt, 2 h; SiO_2, 98%; (v) 1: TfCl, DMAP, CH_2Cl_2, rt, 2 h, CH_2Cl_2/$NaHCO_3$ workup; 2: LiN_3, DMF, rt, overnight; EtOAc/$NaHCO_3$ workup, SiO_2, 85%; (vi) 1: *N*-Fmoc/Ac α-aminoacid or α-hydroxy acid, HOBt, DIC, THF, 0 °C; 2: $(n\text{-}Bu)_3P$, from 0 °C to rt within 30 min, overnight; CH_2Cl_2/$NaHCO_3$ workup, SiO_2, 54–100%; (vii) 1: $NEt_3 \cdot 3HF$ (TBAF for **2e**), THF, rt, overnight; 2: 33% CH_3NH_2 in EtOH, 4 h, rt; EtOAc/CH_2Cl_2/$NaHCO_3$ workup, RP-HPLC.

From a general synthetic point of view, our coupling procedure takes advantage of a classical peptide synthesis activation of carboxylic acids that allows for inexpensive, mild, efficient, and epimerization-free couplings with in situ-generated iminophosphoranes. It demonstrates the compatibility of the Staudinger–Vilarrasa coupling with unprotected phenol and, in part, hydroxyl groups and is the first such protocol to be so simple and high yielding under stoichiometric solution reaction conditions.

2. Results and discussion

2.1. Optimized synthesis of azide 6

We begin the analog's synthesis with the protection the 2′- and 5′-positions as *tert*-butyldimethylsilyl ethers, which gives a mixture of 2′,5′- and 3′,5′-*O*-TBDMS regioisomers. The undesired regioisomer is separated and submitted to isomerizing conditions (2.5% Et_3N in MeOH). A three day-long silylation reaction affords 48% isolated yield of pure 2′,5′-*O*-TBDMS regioisomer. One can rise its overall yield to 85% through two isomerization steps.

The inversion of the configuration at the 3′-position is carried out by an oxidation/reduction sequence. For the oxidation the Garegg reagent proves best (CrO_3 in Ac_2O and pyridine). We found better reduction conditions through the use of commercial $NaHB(OAc)_3$ in CH_3CN containing a minimal amount of AcOH at −18 °C. The much shortened reaction time leads to better yields when compared to the formerly published procedure (90% after 4 h reaction time instead of 84% from $NaBH_4$ in AcOH at 15–16 °C then 12–13 °C after 2.5 days reaction time).[5m] An important advantage is that there is no need for a flash chromatography after the reaction, which proceeds cleanly. A simple workup was found sufficient to obtain crude **5** pure enough for the next step; the small amounts of the non-desired *ribo* isomer (2–5% with respect to **5**) are eliminated during the purification processes of the following steps.

These steps consist in protecting the exocyclic amino group on the adenine moiety (98%), the activation of the 3′-hydroxyl group with help of triflic chloride and the nucleophilic displacement of the triflate intermediate with lithium azide leading to azide **6** (85% yield, scale ≤3 g).

2.2. The synthetic key step: classical amino acid activation combined with optimized Staudinger conditions

Inspired by the pioneering coupling studies on solid support by Tóth and colleagues,[10q] and Piccialli and colleagues,[10r] we developed a coupling protocol that replaces the usual reduction of the organoazide into an amine, which would be the usual substrate for a coupling reaction. The protocol consists in mixing the amino acid derivative (or hydroxy acid) with HOBt (1-hydroxybenzotriazole), DIC (diisopropylcarbodiimide), and **6** at 0 °C. The coupling begins with the dropwise addition of commercial Bu_3P to the reaction mixture and then rising the temperature to ambient within approximately 30 min. Freshly distilled Bu_3P, other coupling conditions—unactivated amino acid, amino acid activated by DEPBT/NMM, DEPBT/DIEA,[12] iminophosphorane formation using Me_3P—[13] other reagents' amounts or a different order of addition[10p] were tested but did not lead to better results.

The reported optimized protocol gives easy access to amides free of residual[14] Bu_3P or Bu_3PO and without partial cleavage of the Fmoc protection[13b] in close to quantitative yields: **7a**, **7b**, **7c**, and **7d** are obtained in 94, 93, 91, and 100 % isolated yields, respectively. Only the yield of **7e** was 54%. For this compound a slight modification of the protocol (evaporation of THF before workup) gives rise to the formation of two by-products as determined by MS: an acyltriazene and a product of β-elimination of HN_3. The combined coupling yields for amide and acyltriazene then reaches 65% and the three final products are detected by ^{1}H NMR with the ratio **2e**/acyltriazene/(2′,3′)-elimination product: 0.68/0.16/0.16.

2.3. Deprotection and purification

With the exception of **2e**, all compounds are deprotected and purified the same way. Desilylation with $NEt_3 \cdot 3HF$ (2.0 equiv) and subsequent deprotection of Fmoc and formamidine groups in 33% ethanolic methylamine. For **2e**, we replaced $NEt_3 \cdot 3HF$ by TBAF (4 equiv).

The final, highly polar compounds were purified by preparative reversed-phase HPLC (eluants: A=10 mM aq NH_4OAc, pH 6.5, B=CH_3CN/H_2O 9:1) and obtained in part (**2a** and **2c**) as lyophilized α-ammonium acetate salts. The products were considered sufficiently pure when the content of acetate shown by ^{1}H NMR was below 5 equiv.

3. Conclusion

Four new puromycin analogs were synthesized for which an optimized nine-step procedure is presented, one that includes a very efficient Staudinger–Vilarrasa coupling reaction that combines standard epimerization-free amino acid activation with optimized Staudinger reaction conditions on organoazides. Mechanistic studies on some aspects of the Staudinger–Vilarrasa coupling are underway. The analogs will be subjected to biological assays.

Many organoazides are usually chemically stable,[15a] readily and stereospecifically accessible and easy to handle (non-hygroscopic, conveniently apolar, and pH-neutral) if, during their synthesis, contact of inorganic azide salts with halogenated solvents[15b–d] and acidification[15e] are avoided. Staudinger reaction conditions are orthogonally compatible with quite a variety of otherwise reducible functional and protecting groups such as, for instance, alkenes, imines, amidines, guanidines, *O*- or *N*-benzyl, *O*-allyl, *N*-allyloxy-, and *N*-benzyloxycarbonyl groups some of which are enjoying widespread usage in nucleoside and peptide syntheses. This version of the Staudinger–Vilarrasa coupling, being a simpler alternative than some others,[16a] opens way to mild and efficient access to other[16b,c] carboxamide target compounds, such as sterically hindered peptides,[16d–h] for instance—one more element that adds to the attractivity of organoazides.[17]

4. Experimental

4.1. General

LiN_3 was prepared from NaN_3 and LiCl (1:1) in EtOH (residual NaN_3 and LiCl were filtered off after treatment with EtOH and then Et_2O); *Caution*: *Do not acidify, do not heat as a dry solid!*[15] ^{1}H NMR spectra (300 MHz) were obtained from solutions in $CDCl_3$, with the residual protonated solvent signal as an internal reference (7.26 ppm for $CHCl_3$). The chemical shifts δ_H are given in ppm; the coupling constants *J* are given in Hertz (Hz); the signals are described as follows: br=broad, s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet. ^{1}H NMR spectra (500 MHz) of the analogs **2a–e** were obtained from solutions in H_2O/D_2O 9:1 using residual acetate as internal standard (1.94 ppm for CH_3COO^-). The water signal at ~4.7 ppm was suppressed using the WET pulse sequence. ^{13}C NMR spectra (125 MHz) were measured in $CDCl_3$; δ_C (central signal)=77.0 ppm. The assignments of ^{1}H- and ^{13}C NMR signals were achieved with the help of D/H exchange, HSQC, COSY, and DEPT experiments if necessary. H(2′) and sometimes H(3′) ^{1}H NMR signals of **2a–e** are close to 4.7 ppm, thus, missing in the corresponding signal listing owing to the water suppression through the WET pulse sequence.

They could be identified by COSY. Mass spectra (MS and HRMS) were obtained using Fast Atomic Bombardment (FAB from CH_2Cl_2 or H_2O/MeOH 9:1) and Electro Spray Ionization (ESI from CH_2Cl_2 or H_2O/MeOH 9:1). Thin Layer Chromatography (TLC) was performed on a precoated silica gel F_{254} plates with fluorescent indicator. The compounds were visualized using UV light (254 nm). Free amines were visualized on TLC plates by spraying with 20% ninhydrin solutions in ethanol, followed by heating. Nucleosides were visualized on TLC plates by subsequent spraying with concentrated H_2SO_4 and 2% naphthoresorcinol solutions in ethanol, followed by heating. Column chromatography was performed with flash silica gel (0.04–0.063 mm).

4.1.1. Preparative RP-HPLC purification. Isocratic conditions were used for the purification of the analogs **2a–e**: A/B 85:15. 250×8 mm Eurospher® 100/5 RP_{18} column, flow rate: 2 mL/min; UV detection at 260 nm. The following eluants were used. A: 10 mM aq NH_4OAc pH ~6.5; B: CH_3CN/H_2O 9:1. Eluants for HPLC were prepared with water purified through the *Milli-Q* system. NH_4OAc was biochemical quality and CH_3CN was HPLC grade.

4.2. Optimized synthetic procedures

4.2.1. Compound 4. See Ref. 5m, reaction time of silylation: 3 days.

4.2.2. 9-[2′,5′-Bis-(*O*-*tert*-butyldimethylsilyl)-β-D-xylofuranosyl]-9*H*-adenine (5). To a suspension of **4** (5 g, 0.010 mol) in dry CH_3CN (200 mL) at room temperature was added just enough AcOH (12 mL) to dissolve all the solid. The mixture was cooled down to −78 °C and $NaHB(OAc)_3$ (2.54 g, 0.012 mol) was added portionwise. After 2 h, the reaction stopped and the same quantity of $NaHB(OAc)_3$ was added to reach total conversion of the material **3**. After 2 more hours, the solution was taken up by 3% H_2O_2/saturated $NaHCO_3/CH_2Cl_2$ (1:100:200 v/v/v) and extracted. The organic layers were combined and washed with saturated $NaHCO_3$ solution and then with H_2O until pH 7–8 was reached. The resulting organic layer was dried over anhydrous $MgSO_4$ and evaporated. Compound **5** (4.46 g, 90%) was obtained as a pale yellow foam. $C_{22}H_{41}N_5O_4Si_2$ (495.77). R_f (AcOEt)=0.49. ^{1}H NMR ($CDCl_3$): δ=0.04, 0.06, 0.07 (3s, 12H, Si–CH_3); 0.87, 0.88 (2s, 18H, Si–C(CH_3)$_3$); 3.98 (dd, 1H, ^3J=5.8 Hz, ^2J=11.3 Hz, H_A(5′)); 4.08 (m, 1H, H(3′)); 4.12 (dd, 1H, ^3J=4.1 Hz, ^2J=11.3 Hz, H_B(5′)); 4.20–4.26 (m, 1H, H(4′)); 4.50 (m, 1H, H(2′)); 5.76 (d, 1H, ^3J=1.2 Hz, H(1′)); 6.18 (br s, 2H, NH_2(6)); 6.72 (br s, 1H, OH(3′)); 7.96 (s, 1H, H(8)); 8.29 (s, 1H, H(2)).[18]

4.2.3. Compound 6. See Ref. 5m.

4.3. General procedure for the coupling of *N*-protected amino acids with the azide 6 to give amides 7a–d

The *N*-protected amino acid (0.096 mmol) was co-evaporated three times with 2 mL freshly distilled THF under reduced pressure and dissolved in 1.75 mL dry THF at 0 °C. To this solution was added HOBt (17 mg, 0.128 mmol). The solution was allowed to stir for 10 min at 0 °C. Then, DIC (20.0 μL, 0.128 mmol) was added dropwise. After having stirred for 10 more minutes at 0 °C, a solution of **6** (49 mg, 0.074 mmol) dissolved in 1.5 mL anhydrous THF was added via syringe, followed by commercial (97%, *Aldrich*) tri-*n*-butylphosphine (40 μL, 0.161 mmol) and the reaction was allowed to pursue overnight with the temperature increasing to rt within approximately 30 min. After evaporation to dryness, the solid was then taken up with 0.7 mL water. The product was extracted three times with 15 mL CH_2Cl_2. The combined organic layers were washed by 35 mL saturated $NaHCO_3$ solution and 35 mL water, dried over anhydrous Na_2SO_4, filtered, and evaporated. Purification by flash chromatography (EtOAc/hexanes 1:1) gave **7a–e** as colorless oils.

4.3.1. 6-*N*-[(Di-*n*-butylamino)methylene]-2′,5′-bis-(*O*-*tert*-butyldimethylsilyl)-3′-[α-*N*-(9-fluorenyl)methoxycarbonyl-(*p*-methoxy-L-phenylalanyl)amino]-3′-deoxyadenosine (7a). Yield: 67 mg, 94%. $C_{56}H_{80}N_8O_7Si_2$ (1033.48). R_f (AcOEt/petrol ether 50:50)=0.5. ^{1}H NMR ($CDCl_3$): δ=−0.14, −0.13, 0.14 (3s, 12H, Si–CH_3); 0.74 (s, 9H, Si–C(CH_3)$_3$); 0.95 (m, 15H, Si–C(CH_3)$_3$, N($CH_2CH_2CH_2CH_3$)$_2$); 1.31–1.45 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 1.59–1.71 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 2.83–2.94 (br, 1H, H(β1)-*O*–MeTyr); 3.10–3.20 (br m, 1H, H(β2)-*O*-MeTyr); 3.38 (t, 2H, ^3J=7.2 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.70 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$); 3.77 (s, 3H, CH_3-*O*-MeTyr); 3.83–3.99 (m, 3H, H(5′), H(9″)-Fmoc); 4.20 (t, 1H, ^3J=6.7 Hz, H(α)-*O*-MeTyr); 4.30–4.50 (m, 4H, H(4′), H(3′), (*O*-CH_2)–Fmoc); 4.57 (t, 1H, ^3J=6.7 Hz, 5.0 Hz, H(2′)); 5.45 (br, 1H, NH-carbamate); 5.90 (d, 1H, ^3J=3.7 Hz, H(1′)); 6.08 (br, 1H, NH-amide), 6.88 (d, 2H, ^3J=8.5 Hz, H arom.-Tyr); 7.11 (br, 2H, H arom.-*O*-MeTyr); 7.30 (t, 2H, ^3J=7.5 Hz, H(2″)); 7.39 (t, 2H, ^3J=7.5 Hz, H(3″)); 7.55 (dd, 2H, ^4J=3.4 Hz, ^3J=7.5 Hz, H(1″)); 7.76 (d, 2H, ^3J=7.5 Hz, H(4″)); 8.26 (s, 1H, H(8)); 8.50 (s, 1H, H(2)); 8.98 (s, 1H, N^6=CH).[18]

4.3.2. 3′-[α-*N*-Acetyl-(*p*-methoxy-L-phenylalanyl)amino]-6-*N*-[(di-*n*-butylamino)methylene]-2′,5′-bis-(*O*-*tert*-butyldimethylsilyl)-3′-deoxyadenosine (7b). Yield: 59 mg, 93%. $C_{43}H_{72}N_8O_6Si_2$ (853.26). R_f (AcOEt)=0.48. ^{1}H NMR ($CDCl_3$): δ=−0.10, 0.14 (2s, 12H, Si–CH_3); 0.78 (s, 9H, Si–C(CH_3)$_3$); 0.96 (s, 15H, Si–C(CH_3)$_3$, N($CH_2CH_2CH_2CH_3$)$_2$); 1.38 (hex, 4H, ^3J=7.6 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 1.59–1.71 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 2.00 (s, 3H, CH_3–Ac); 2.80 (dd, 1H, ^3J=9.2 Hz, ^2J=13.7 Hz, H(β1)-*O*-MeTyr); 3.14 (dd, 1H, ^3J=5.1 Hz, ^2J=13.7 Hz; H(β2)-*O*-MeTyr); 3.40 (t, 2H, ^3J=7.3 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.71 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$); 3.77 (s, 3H, CH_3-*O*-MeTyr); 3.78–3.82 (m, 2H, H(5′)); 3.90 (m, 1H, H(4′)); 4.42 (q, 1H, ^3J=6.1 Hz, H(3′)); 4.48–4.57 (m, 2H, H(α)-*O*-MeTyr, H(2′)); 5.85 (d, 1H, ^3J=4.0 Hz, H(1′)); 5.89 (d, 1H, ^3J=6.1 Hz, NH–C(3′)); 6.21 (d, 1H, ^3J=7.2 Hz, NH–Ac); 6.88, 7.15 (2d, 4H, ^3J=8.6 Hz, H arom.-*O*-MeTyr); 8.27 (s, 1H, H(8)); 8.52 (s, 1H, H(2)); 9.04 (s, 1H, N^6=CH). ^{13}C NMR ($CDCl_3$): δ=−5.32 (Si–CH_3); 13.70, 13.95 (N($CH_2CH_2CH_2CH_3$)$_2$); 17.81, 18.50 (Si–*C*(CH_3)$_3$); 19.77, 20.20 (N($CH_2CH_2CH_2CH_3$)$_2$); 22.77 (CH_3–Ac); 25.58, 26.07 (Si–C(*C*H_3)$_3$); 29.29, 32.04 (N($CH_2CH_2CH_2CH_3$)$_2$); 39.04 (C(β)-*N*-AcTyr); 45.37 (N($CH_2CH_2CH_2CH_3$)$_2$); 50.79 (C(3′)); 52.19 (N($CH_2CH_2CH_2CH_3$)$_2$); 55.13 (C(α)-*O*-MeTyr); 55.29 (CH_3-*O*-MeTyr); 63.34 (C(5′)); 76.08 (C(2′)); 84.82 (C(4′));

89.2 (C(1′)); 92.72 (C arom.-*O*-MeTyr); 114.33 (C arom.-*O*-MeTyr); 126.07 (C(5)); 130.20 (C arom.-*O*-MeTyr); 139.95 (C(8)); 150.47 (C(4)); 153.06 (C(2)); 155.51 (C arom.-*O*-MeTyr); 157.26 (C(6)); 158.83 (N^6=CH(*n*-Bu)$_2$); 169.63, 170.83 (amide C=O). HRMS (ESI$^+$): Exact mass calculated for $C_{43}H_{73}N_8O_6Si_2$: 853.5191. Found: 853.5196.

4.3.3. 6-*N*-[(Di-*n*-butylamino)methylene]-2′,5′-bis-(*O*-*tert*-butyldimethylsilyl)-3′-[α-*N*-(9-fluorenyl)methoxycarbonyl-(*p*-hydroxy-L-phenylalanyl)amino]-3′-deoxyadenosine (7c). Yield: 69 mg, 91%. $C_{55}H_{78}N_8O_7Si_2$ (1019.44). R_f (AcOEt/petrol ether 50:50)=0.31. ^{1}H NMR ($CDCl_3$): δ=−0.07, −0.06, 0.11, 0.12 (4s, 12H, Si–CH_3); 0.75 (s, 9H, Si–C(CH_3)$_3$); 0.94 (s, 15H, Si–C(CH_3)$_3$, N($CH_2CH_2CH_2CH_3$)$_2$); 1.33–1.44 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 1.61–1.72 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 2.83 (m, 1H, H(β1)-*N*-FmocTyr); 2.91–2.97 (m, 1H, H(β2)-*N*-FmocTyr); 3.43 (t, 2H, ^3J=7.2 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.67 (t, 2H, ^3J=7.6 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.71–3.77 (m, 2H, H(5′)); 3.93–4.00 (m, 1H, H(4′)); 4.48 (t, 1H, ^3J=5.3 Hz, H(α)-*N*-FmocTyr); 4.56 (t, 1H, ^3J=6.2 Hz, H(3′)); 4.67–4.72 (m, 1H, H(2′)); 6.03 (d, 1H, ^3J=3.3 Hz, H(1′)); 6.70, 7.05 (2d, 4H, ^3J=8.1 Hz, H arom.-*N*-FmocTyr); 7.21–7.26 (m, 2H, H(2″)); 7.32 (t, 2H, ^3J=7.4 Hz, H(3″)); 7.53–7.56 (m, 2H, H(1″)); 7.76 (d, 2H, ^3J=7.5 Hz, H(4″)); 8.41 (s, 1H, H(8)); 8.47 (s, 1H, H(2)); 8.94 (s, 1H, N^6=CH). ^{13}C NMR ($CDCl_3$): δ=−5.46, −5.28 (Si–CH_3); 13.65, 13.66 (N($CH_2CH_2CH_2CH_3$)$_2$); 17.61, 18.40 (Si–*C*(CH_3)$_3$); 19.77, 20.18 (N($CH_2CH_2CH_2CH_3$)$_2$); 26.06, 26.09 (Si–C(*C*H_3)$_3$); 29.70, 30.95 (N($CH_2CH_2CH_2CH_3$)$_2$); 42.27 (C(β)-Tyr); 45.69 (N($CH_2CH_2CH_2CH_3$)$_2$); 47.21 (C(9″)-Fmoc); 51.92 (N($CH_2CH_2CH_2CH_3$)$_2$); 52.98 (C(α)-*N*-FmocTyr); 57.25 (C(3′)); 63.10 (C(5′)); 67.03 ((*O*-CH_2)–Fmoc); 74.91 (C(2′)); 85.11 (C(4′)); 86.79 (C(1′)); 119.17 (C arom.-*N*-FmocTyr); 120.00, 125.13 (C arom.-*N*-Fmoc); 126.00 (C(5)); 127.08, 127.75 (C arom.-*N*-Fmoc); 129.00, 130.12 (C arom.-*N*-FmocTyr); 139.43 (C(8)); 141.32, 143.79 (C arom.-*N*-Fmoc); 150.95 (C(4)); 152.42 (C(2)); 155.92 (C arom.-*N*-FmocTyr); 156.96 (C(6)); 158.47 (N^6=CH(*n*-Bu)$_2$); 160.64 (carbamate C=O); 170.28 (amide C=O). HRMS (ESI$^+$): Exact mass calculated for $C_{55}H_{79}N_8O_7Si_2$: 1019.5610. Found: 1019.5616.

4.3.4. 3′-[α-*N*-Acetyl-(*p*-hydroxy-L-phenylalanyl)amino]-6-*N*-[(di-*n*-butylamino)methylene]-2′,5′-bis-(*O*-*tert*-butyldimethylsilyl)-3′-deoxyadenosine (7d). Yield: 62 mg, 100%. $C_{42}H_{70}N_8O_6Si_2$ (839.23). R_f (AcOEt/petrol ether 70:30)=0.82. ^{1}H NMR ($CDCl_3$): δ=−0.37, −0.21, 0.12, 0.13 (4s, 12H, Si–CH_3); 0.71 (s, 6H, N($CH_2CH_2CH_2CH_3$)$_2$); 0.94 (s, 18H, Si–C(CH_3)$_3$); 1.31–1.44 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 1.59–1.72 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 2.03 (s, 3H, CH_3–Ac); 3.37–4.51 (m, 9H, N($CH_2CH_2CH_2CH_3$)$_2$, H(5′), H(α)-*N*-AcTyr, H(4′), H(3′)); 4.54 (t, 1H, ^3J=6.9 Hz, H(2′)); 5.39 (d, 1H, ^3J=3.7 Hz, NH–C(3′)); 5.60 (d, 1H, ^3J=6.9 Hz, H(1′)); 6.52 (d, 1H, ^3J=7.1 Hz, NH–Ac); 7.06, 7.17 (2d, 4H, ^3J=8.3 Hz, H arom.-*N*-AcTyr); 8.23 (s, 1H, H(8)); 8.59 (s, 1H, H(2)); 8.98 (s, 1H, N^6=CH). ^{13}C NMR ($CDCl_3$): δ=−5.74 (Si–CH_3); 13.56, 13.80 (N($CH_2CH_2CH_2CH_3$)$_2$); 17.43, 18.22 (Si–*C*(CH_3)$_3$); 19.57, 19.97 (N($CH_2CH_2CH_2CH_3$)$_2$); 23.27 (CH_3–Ac); 25.13, 25.84 (Si–C(*C*H_3)$_3$); 28.98, 30.66 (N($CH_2CH_2CH_2CH_3$)$_2$); 39.14 (C(β)-*N*-AcTyr); 45.01 (N($CH_2CH_2CH_2CH_3$)$_2$); 51.75 (N($CH_2CH_2CH_2CH_3$)$_2$); 52.76 (C(α)-*N*-AcTyr); 55.50 (C(3′)); 63.98 (C(5′)); 74.61 (C(2′)); 84.82 (C(4′)); 86.50 (C(1′)); 93.00 (C arom.-*N*-AcTyr); 118.93 (C arom.-*N*-AcTyr); 125.65 (C(5)); 129.87 (C arom.-*N*-AcTyr); 139.26 (C(8)); 150.53 (C(4)); 152.11 (C(2)); 155.65 (C arom.-*N*-AcTyr); 156.81 (C(6)); 158.25 (N^6=CH(*n*-Bu)$_2$); 169.27, 170.16 (amide C=O). HRMS (ESI$^+$): Exact mass calculated for $C_{42}H_{71}N_8O_6Si_2$: 839.5035. Found: 839.5034.

4.3.5. (2*S*)-6-*N*-[(Di-*n*-butylamino)methylene]-2′,5′-bis-(*O*-*tert*-butyldimethylsilyl)-3′-[(2-hydroxy-3-phenylpropionyl)amino]-3′-deoxyadenosine (7e). To a solution of L-phenyllactic acid (126 mg, 0.76 mmol) in 6 mL freshly distilled dry THF at 0 °C was added HOBt (97 mg, 0.72 mmol). The solution was allowed to stir for 10 min at 0 °C. Then, DIC (115.8 μL, 0.74 mmol) was added dropwise. After having stirred for 10 more minutes at 0 °C, a solution of **6** (241 mg, 0.37 mmol) dissolved in 6 mL freshly distilled anhydrous THF was added via syringe, followed by tri-*n*-butylphosphine (180 μL, 0.70 mmol) and the reaction was allowed to pursue overnight with a temperature increasing until rt within approximately 30 min. After evaporation to dryness, the solid was then taken up by 3 mL water. The product was extracted three times with 100 mL CH_2Cl_2. The organic layers were washed by 150 mL saturated $NaHCO_3$ solution and 150 mL water, dried over anhydrous $MgSO_4$, filtered, and evaporated. Purification by flash chromatography (EtOAc/hexanes 1:1) gave **7e** as a colorless oil. Yield: 321 mg, 54%. $C_{40}H_{67}N_7O_5Si_2$ (782.17). R_f (AcOEt/petrol ether 50:50)=0.29. ^{1}H NMR ($CDCl_3$): δ=−0.03, −0.01 (m, 6H, Si–CH_3); 0.15–0.19 (m, 6H, Si–CH_3); 0.85 (m, 9H, Si–C(CH_3)$_3$); 0.96 (m, 15H, Si–C(CH_3)$_3$, N($CH_2CH_2CH_2CH_3$)$_2$); 1.65 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 1.36 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$); 2.84 (dd, 1H, ^3J=9.1 Hz, ^2J=13.9 Hz, Hβ1); 3.26 (dd, 1H, ^3J=3.6 Hz, ^2J=13.9 Hz, Hβ2); 3.39 (t, 2H, ^3J=7.2 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.72 (t, 2H, ^3J=7.6 Hz, N($CH_2CH_2CH_2CH_3$)$_2$); 3.74 (m, 1H, H(5′)); 4.10 (m, 1H, Hα); 4.20 (m, 1H, H(4′)); 4.22 (m, 1H, H(3′)); 4.61 (m, 1H, H(2′)); 6.07 (d, 1H, ^3J=3.7 Hz, H(1′)); 7.18–7.33 (m, 5H, H arom.-L-phenyllactic acid); 8.31 (s, 1H, H(8)); 8.54 (s, 1H, H(2)); 9.01 (s, 1H, N^6=CH). ^{13}C NMR ($CDCl_3$): δ=−5.38, −5.31, −5.06, −4.97 (Si–CH_3); 13.67, 13.91 (N($CH_2CH_2CH_2CH_3$)$_2$); 17.82, 18.38 (Si–*C*(CH_3)$_3$); 19.76, 20.17 (N($CH_2CH_2CH_2CH_3$)$_2$); 25.54, 26.01 (Si–C(*C*H_3)$_3$); 29.18, 30.91 (N($CH_2CH_2CH_2CH_3$)$_2$); 45.02 (N($CH_2CH_2CH_2CH_3$)$_2$); 50.70 (C(3′)); 40.98 (C(β)); 51.77 (N($CH_2CH_2CH_2CH_3$)$_2$); 64.10 (C(5′)); 76.02 (C(2′)); 82.09 (CH(α)); 84.37 (C(4′)); 88.89 (C(1′)); 125.89 (C(5)); 128.56, 128.77, 129.41, 129.58 (C arom.-L-phenyllactic acid); 140.20 (C(8)); 151.19 (C(4)); 152.68 (C(2)); 158.32 (N^6=CH(*n*-Bu)$_2$); 160.10 (C(6)); 171.09 (amide C=O). HRMS (ESI$^+$): Exact mass calculated for $C_{40}H_{68}N_7O_5Si_2$: 782.4821. Found: 782.4823.

4.4. General procedure for the deprotection of analogs 7a–d to give 3′-(α-amino/*N*-acetylamidoacyl)amino-3′-deoxyadenosines 2a–d

To a solution of **7a–d** (0.08 mmol) in THF (1.0 mL) was added $Et_3N\cdot 3HF$ (~37% HF, 25 μL, 0.15 mmol). The solution was stirred at rt overnight. The THF was evaporated on a rotatory evaporator under reduced pressure and a solution (1.0 mL) of 33% (v/v) methylamine in EtOH was added. The mixture was stirred at rt for 4 h, taken up by 3 mL water.

Organic impurities were extracted successively three times with 7 mL EtOAc and three times with 7 mL CH_2Cl_2. The combined organic layers were extracted successively with 3 mL $NaHCO_3$, then 3 mL water. The combined aqueous phases were concentrated, re-diluted with HPLC eluant A, filtered (0.7 μm) and injected to purify by RP-HPLC. The fractions containing **2a–d** were concentrated in a $Si(CH_3)_2Cl_2$-treated glass flask on a rotatory evaporator under reduced pressure, then repeatedly lyophilized in Eppendorf tubes under 'high-vacuum' (SpeedVac) until NH_4OAc was present in lower than 5 molar equivalent amounts with respect to the analogs **2a–d**, as determined by ^1H NMR. The analogs **2a–d** were thus isolated as white fluffy solids.

4.4.1. 3′-(*p*-Methoxy-L-phenylalanylamino)-3′-deoxyadenosine (2a). ^1H NMR (H_2O/D_2O 9:1): δ=3.00–3.10 (m, 1H, H(β1)-*O*-MeTyr); 3.27 (dd, 1H, ^3J=6.1 Hz, ^2J=13.9 Hz, H(β2)-*O*-MeTyr); 3.45 (dd, 1H, ^3J=3.8 Hz, ^2J=13.4 Hz, $H_A(5')$); 3.71 (dd, 1H, ^3J=1.6 Hz, ^2J=13.4 Hz, $H_B(5')$); 3.84 (s, 3H, CH_3-*O*-MeTyr); 3.85–3.88 (m, 1H, H(4′)); 4.24 (dd, 1H, $^3J_{H(\alpha)-H(\beta2)}$=6.9 Hz, $^3J_{H(\alpha)-H(\beta1)}$=10.2 Hz, H(α)-*O*-MeTyr); 5.96 (d, 1H, $^3J_{H(1')-H(2')}$=3.6 Hz, H(1′)); 7.05, 7.28 (2d, 4H, ^3J=8.5 Hz, H arom.-*O*-MeTyr); 8.26 (s, 1H, H(2)); 8.34 (s, 1H, H(8)).[18]

4.4.2. 3′-[α-*N*-Acetyl-(*p*-methoxy-L-phenylalanylamino)]-3′-deoxyadenosine (2b). $C_{22}H_{27}N_7O_6$ (485.49). ^1H NMR (H_2O/D_2O 9:1): δ=1.86 (s, 3H, CH_3–Ac); 2.84–2.88 (m, 1H, H(β1)-*N*-AcTyr); 3.00 (dd, 1H, ^3J=7.1 Hz, ^2J=14.7 Hz, H(β2)-*N*-AcTyr); 3.32–3.37 (m, 2H, H(5′)); 3.69 (s, 3H, CH_3-*O*-MeTyr); 3.78 (m, 1H, H(4′)); 5.83 (d, 1H, $^3J_{H(1')-H(2')}$=2.2 Hz, H(1′)); 6.86, 7.12 (2d, 4H, ^3J=8.4 Hz, H arom.-*O*-MeTyr); 8.11 (s, 1H, H(2)); 8.21 (s, 1H, H(8)). HRMS (ESI^+): Exact mass calculated for $C_{22}H_{28}N_7O_6$: 486.2101. Found: 486.2106.

4.4.3. 3′-(*p*-Hydroxy-L-phenylalanylamino)-3′-deoxyadenosine (2c). $C_{19}H_{23}N_7O_5$ (429.43). ^1H NMR (H_2O/D_2O 9:1): δ=2.67–2.73 (m, 1H, H(β1)-Tyr); 2.95 (dd, 1H, ^3J=6.2 Hz, ^2J=13.1 Hz, H(β2)-Tyr); 3.29 (dd, 1H, ^3J=3.9 Hz, ^2J=13.0 Hz, $H_A(5')$); 3.56 (dd, 1H, ^3J=1.7 Hz, ^2J=13.0 Hz, $H_B(5')$); 3.69 (m, 1H, H(4′)); 3.77 (m, 1H, H(3′)); 4.38 (dd, 1H, $^3J_{H(\alpha)-H(\beta2)}$=6.2 Hz, $^3J_{H(\alpha)-H(\beta1)}$=7.6 Hz, H(α)-Tyr); 5.88 (d, 1H, $^3J_{H(1')-H(2')}$=3.1 Hz, H(1′)); 6.80, 7.07 (2d, 4H, ^3J=8.6 Hz, H arom.-Tyr); 8.15 (s, 1H, H(2)); 8.23 (s, 1H, H(8)). HRMS (ESI^+): Exact mass calculated for $C_{19}H_{24}N_7O_5$: 430.1839. Found: 430.1829.

4.4.4. 3′-[α-*N*-Acetyl-(*p*-hydroxy-L-phenylalanylamino)]-3′-deoxyadenosine (2d). $C_{21}H_{25}N_7O_6$ (471.47). ^1H NMR (H_2O/D_2O 9:1): δ=1.98 (s, 3H, CH_3–Ac); 2.87 (dd, 1H, ^3J=9.3 Hz, ^2J=13.7 Hz, H(β1)-*N*-AcTyr); 3.02 (dd, 1H, ^3J=7.0 Hz, ^2J=13.7 Hz, H(β2)-*N*-AcTyr); 3.41 (dd, 1H, ^3J=4.0 Hz, ^2J=13.0 Hz, $H_A(5')$); 3.66 (m, 1H, $H_B(5')$); 3.87–3.90 (m, 1H, H(4′)); 4.46 (dd, 1H, $^3J_{H(3')-H(2')}$=5.9 Hz, $^3J_{H(3')-H(4')}$=7.8 Hz, H(3′)); 4.53 (dd, 1H, $^3J_{H(\alpha)-H(\beta2)}$=7.0 Hz, $^3J_{H(\alpha)-H(\beta1)}$=9.3 Hz, H(α)-*N*-AcTyr); 4.68 (dd, 1H, $^3J_{H(2')-H(1')}$=3.1 Hz, $^3J_{H(2')-H(3')}$=5.9 Hz, H(2′)); 5.98 (d, 1H, $^3J_{H(1')-H(2')}$=3.1 Hz, H(1′)); 6.78, 7.11 (2d, 4H, ^3J=8.5 Hz, H arom.-*N*-AcTyr); 8.22 (s, 1H, H(2)); 8.31 (s, 1H, H(8)). HRMS (ESI^+): Exact mass calculated for $C_{21}H_{26}N_7O_6$: 472.1944. Found: 472.1947.

4.4.5. (2*S*)-3′-[(2-Hydroxy-3-phenylpropionyl)amino]-3′-deoxyadenosine (2e). To a solution of **7e** (0.08 mmol) in THF (1.0 mL) was added TBAF (320 μL, 0.32 mmol). The solution was stirred at rt for 2 h. THF was evaporated on a rotatory evaporator under reduced pressure and a solution (1.0 mL) of 33% (v/v) methylamine in EtOH was added. The mixture was stirred at rt for 4 h and then taken up by 3 mL water. Organic impurities were extracted successively three times with 7 mL EtOAc and three times with 7 mL CH_2Cl_2. The organic layers were both washed by 3 mL $NaHCO_3$, then 3 mL water. The aqueous phase was directly purified by RP-HPLC with use of the same conditions as described for the analogs **2a–d**. The fractions containing **2e** were concentrated in a $Si(CH_3)_2Cl_2$-treated glass flask on a rotary evaporator under reduced pressure, then repeatedly lyophilized in Eppendorf tubes under 'high-vacuum' (SpeedVac), until NH_4OAc was present in lower than 5 molar equivalent amounts with respect to the analog **2e**, as determined by ^1H NMR. The analog **2e** was thus isolated as white fluffy solid. $C_{19}H_{22}N_6O_5$ (414.42). ^1H NMR (H_2O/D_2O 9:1): δ=2.95 (dd, 1H, ^3J=4.1 Hz, ^2J=8.1 Hz, H(β1)-L-3-phenyllactic acid); 3.00 (dd, 1H, ^3J=4.1 Hz, ^2J=8.1 Hz, H(β2)-L-3-phenyllactic acid); 3.50 (dd, 1H, ^3J=4.3 Hz, ^2J=13.5 Hz, $H_A(5')$); 3.70 (dd, 1H, ^3J=2.4 Hz, ^2J=13.5 Hz, $H_B(5')$); 3.96–4.02 (m, 1H, H(4′)); 4.41–4.49 (m, 2H, H(3′), H(α)-L-3-phenyllactic acid); 4.52–4.57 (m, 1H, H(2′)); 5.96 (d, 1H, $^3J_{H(1')-H(2')}$=2.8 Hz, H(1′)); 7.16–7.33 (m, 5H, H arom.-L-3-phenyllactic acid); 8.15 (s, 1H, H(2)); 8.25 (s, 1H, H(8)). HRMS (ESI^+): Exact mass calculated for $C_{19}H_{23}N_6O_5$: 415.1730. Found: 415.1730.

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