the stock solvent does not significantly affect the conformational properties of the peptides. For example, when the lyophilized powder of 7.EKEK was directly dissolved in the buffer, that is, without TFE, the peptide initially formed an α-helical structure which gradually changed to the β-sheet fibrils, as was the case when using TFE as the stock solvent. Thus, the initial α-helix structure is not induced by TFE. For quantitative sample preparations, TFE stock solutions were used. CD spectra were measured by using a quartz cell with 1.0 mm path length, and recorded in terms of mean residual molar ellipticity ($[\theta]$ deg cm^2 dmol^{-1}).

Thioflavin T(ThT)-binding analysis: Peptide solutions were prepared and incubated as described for the CD measurements. After the indicated incubation periods, the ThT solution (240 μM in water) was added to the peptide solution, after which fluorescence measurements were carried out.[7, 9] For the measurements shown in Figure 1, fluorescence emission spectra of solutions of 6 μM ThT in the presence of 12 μM peptide were recorded at an excitation wavelength of 440 nm by using a 5 × 5 mm quartz cell. For the measurements shown in Figure 2a, fluorescence intensities of solutions of 20 μM ThT in the presence of 11 μM peptide (final volume 330 μL) were recorded on a multi-well plate reader (excitation filter, 425 – 475 nm; emission filter, 525 – 535 nm) using a 96-well plate.

TEM study: Peptide solutions were prepared and incubated as described for the CD measurements. The sample was adsorbed to a carbon-coated copper grid and then negatively stained with a 2% (w/v) aqueous phosphotungstic acid solution.

We are grateful to Dr. Yoshio Iwasaki (Rigaku Corporation) for the X-ray diffraction measurement. Y.T. is a Research Fellow of the Japan Society for the Promotion of Science. We thank Ms. Kim Pepin for critically reading the manuscript.

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[11] Although ThT in the presence of 1.EEEE showed a little fluorescence enhancement, similar in degree to that observed for 11.KKEE (Figure 1), CD and TEM studies revealed that 1.EEEE was predominantly present in the random-coil conformation during the observation period and that there was no fibrous aggregate. Thus, we concluded that 1.EEEE was not capable of forming β-sheet fibrils. In contrast, 11.KKEE was shown to form fibrils by TEM analysis, although its CD spectrum after 4 d incubation was not typical for a β-sheet structure and the fluorescence intensity of ThT was not very high.

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Selective Cleavage of Unpaired Uridines with a Tyrosine – Cyclen Conjugate

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KEYWORDS:

hydrolyses · macrocycles · nucleosides · peptides · RNA recognition

Small molecules that selectively cleave RNA could not only act as important tools in molecular biology, they also have the potential for interfering with the life cycle of cells. These compounds could be developed into therapeutic agents. In this context we described the selective cleavage of the transactivation response element of HIV-1 RNA (TAR RNA) by the conjugate **1** in which the arginine-rich region of the transactivator protein Tat is covalently attached to cyclen (1,4,7,10-tetraazacyclododecane).[1] The only cleavage site was located between bases U31 and G32, as can be seen by comparing the results of the gel electrophoresis after alkaline hydrolysis and RNase T1 digestion with those of the cleavage by **1** (Figure 1a; lanes 6, 4, and 1, respectively). In the course of mechanistic investigations of the

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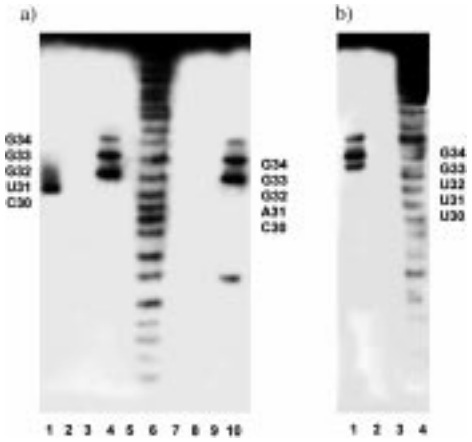
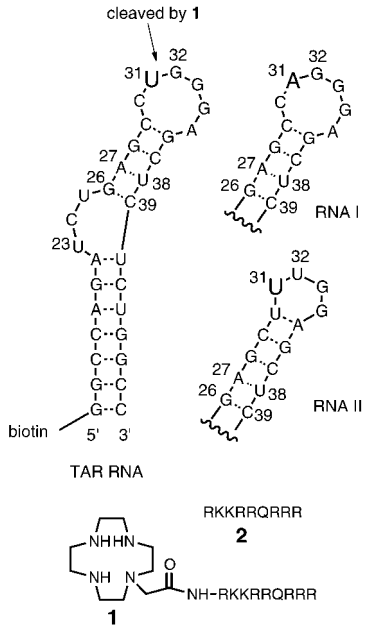


Figure 1. *Result of polyacrylamide gel electrophoresis (PAGE) after sequence-selective hydrolysis of different RNAs (biotin-labeled at the 5′ end) by* ***1****. a) Hydrolysis of TAR RNA and RNA I at room temperature and pH 7.4. Lanes 1 – 6: cleavage of TAR RNA; lane 1: incubation with* ***1*** *for 1 h; lane 2: control; lane 3: incubation with* ***2*** *for 1 h; lane 4: hydrolysis by RNAse T1 (G-specific); lane 5: control; lane 6: alkaline hydrolysis. Lanes 7 – 10: cleavage of RNA I; lane 7: incubation with* ***1*** *for 1 h; lane 8: incubation with* ***2*** *for 1 h; lane 9: control; lane 10: hydrolysis by RNAse T1. b) Hydrolysis of RNA II. Lane 1: hydrolysis by RNAse T1; lane 2: control; lane 3: incubation with* ***1*** *for 1 h; lane 4: alkaline hydrolysis.*

unexpected yet remarkable cleavage reaction we investigated the dependence of this particular cleavage on the type of nucleobase. To assess the role of U31 in the cleavage reaction we changed U31 to A31 in one RNA (RNA I) and the contiguous residues C30 and G32 to uridines (→U30 and U32) in a second RNA (RNA II). As a consequence, an additional base pair (U30·A35) was introduced, which changed the loop to a four-base loop, in contrast to the six-residue loop present in wild-type TAR RNA (Scheme 1).

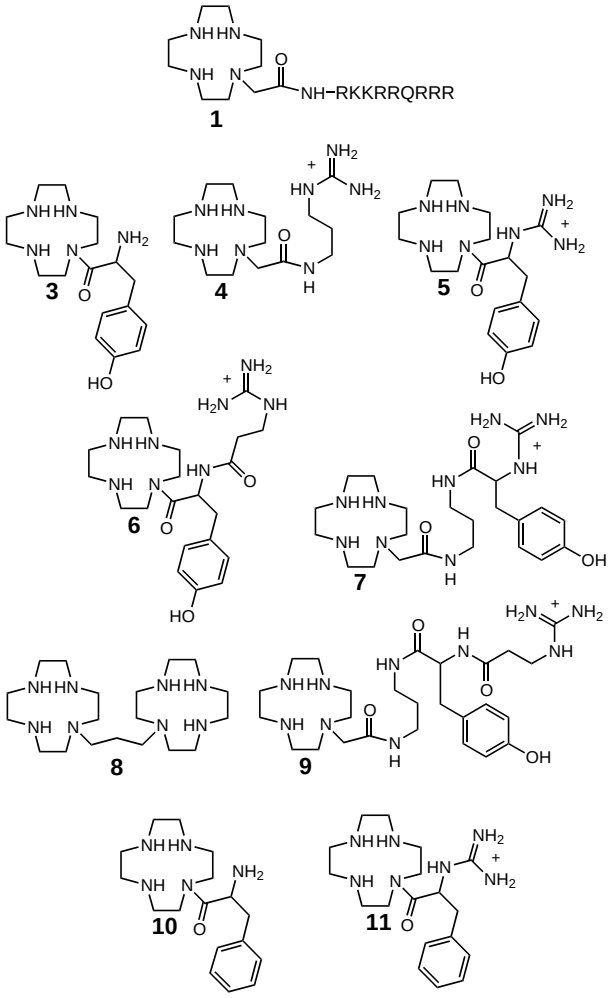


Scheme 1. *TAR RNA and modified RNAs (RNA I and RNA II). The 9-mer – cyclen conjugate* ***1*** *and the arginine-rich region of the Tat protein (peptide* ***2****) are also shown.*

Remarkably, no cleavage was observed when U31 was changed to A31 (Figure 1a, lane 7). Even with three uridine residues in the loop of the RNA (RNA II), no cleavage could be observed in the presence of **1** (Figure 1b, lane 3). This was particularly surprising since U31, at which the cleavage was observed originally, was still in place.

At this point we rationalized that the observed selective cleavage of the wild-type TAR RNA was due to the known specific interaction of the 9-mer peptide of **1** with the TAR RNA[2] and an interaction between the cyclen moiety and uracil.[3] The absence of any cleavage when two unpaired uridines were present in the loop of the RNA could then be explained by an unfavorable location of the cyclen moiety due to the selective binding of the 9-mer peptide in the bulge region of the RNA. With this hypothesis in mind, we envisioned that a small library of tyrosine – cyclen conjugates (Scheme 2) could provide a derivative that exhibits unspecific binding to RNA and cleaves at uridines.

We chose tyrosine for the construction of the cyclen conjugates since this amino acid could facilitate binding by interactions of its side chain and increase the cleavage reaction by providing an additional nucleophilic group.[4] In order to



Scheme 2. *The tyrosine – cyclen derivatives and the cyclen dimer* ***8****.*

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increase the affinity for RNA we decided to transform one amino group of these derivatives into a guanidinium group. Additionally, a dimeric cyclen derivative (**8**) and a cyclen moiety containing only an amino acid spacer and one terminal guanidinium group (**4**) were synthesized for this purpose. Remarkably, only the tyrosine derivative **5**[5] cleaved the TAR RNA (Figure 2, lane 4). The analysis of the cleavage reaction showed not only cleavage at U31, which was observed before with the peptide–cyclen complex **1**, but also in the bulge at position U25. Neither derivative **4** nor the dimeric cyclen compound **8** gave any cleavage.

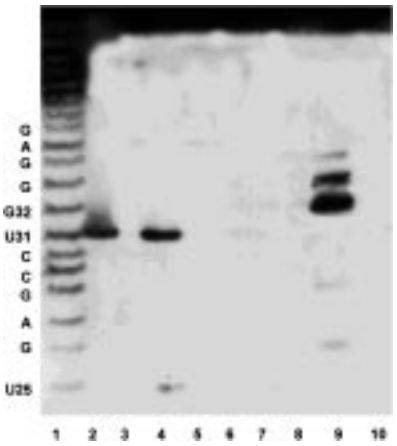

Figure 2. *PAGE result after sequence-selective hydrolysis of TAR RNA by cyclen derivatives at room temperature and pH 7.4. Lane 1: alkaline hydrolysis; lane 2: incubation with **1** for 1 h; lane 3: incubation with **3** for 1 h; lane 4: incubation with **5** for 1 h; lane 5: incubation with **6** for 1 h; lane 6: incubation with **7** for 1 h; lane 7: incubation with **8** for 1 h; lane 8: incubation with **9** for 1 h; lane 9: hydrolysis by RNAse T1; lane 10: incubation with **4** for 1 h.*

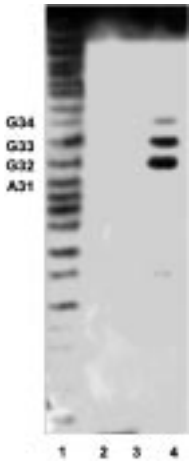

Figure 3. *PAGE result after sequence-selective hydrolysis of RNA I by **5**. Lane 1: alkaline hydrolysis; lane 2: control; lane 3: incubation with **5** for 1 h; lane 4: hydrolysis by RNAse T1.*

We took this as an indication that the guanidinium group serves as the source for a positive charge that increases the unspecific binding of these compounds to the RNA. The results of the cleavage of the modified TAR RNAs with compound **5** supported our hypothesis. With RNA I, in which the only uridine residue in the loop was changed to adenosine, again no cleavage was observed (Figure 3). On the other hand, incubation with RNA II, in which two additional residues had been changed, two spots indicated cleavage at the unpaired uridine residues U31 and U32 (Figure 4).

At this stage it seemed as if the tyrosine–cyclen conjugate **5** could serve as a new tool for the selective cleavage of RNA, with a preference for unpaired uridines. To further evaluate the potential of **5** we performed cleavage reactions with two aptamer RNAs, which had been described as binders for the 17-mer peptide fragment of the HIV-1 Rev protein (Figures 5 and 7).[6] These RNAs contain unpaired regions with uridines at different positions. The reaction of aptamer I with the cyclen derivative **5** resulted in cleavage at positions U7, U12, U15, and U18, and, to a lesser extent, at C14. No cleavage was observed at purine residues (Figure 5).

At this point we had to investigate whether the tyrosine residue was essential for the cleavage, or if phenylalanine analogues would also give the same results. Additionally, we wanted to confirm the mode of action of the cleavage reaction in which the 2′-hydroxy group would be essential for the transesterification. Finally, ribothymidine (T) was used as the base to see if a small modification of the uridine would be tolerated in the cleavage reaction. Figure 6 shows the results of cleaving the modified aptamer I (aptamer Ib) with **5** and the phenylalanine analogues **10** and

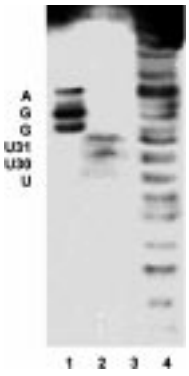

Figure 4. *PAGE result (15% (w/v) polyacrylamide) after sequence-selective hydrolysis of RNA I by **5**. Lane 1: hydrolysis by RNAse T1; lane 2, incubation with **5** for 1 h; lane 3: control; lane 4: alkaline hydrolysis.*

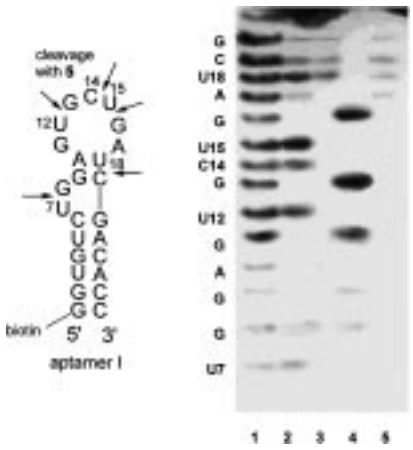

Figure 5. *PAGE result after sequence-selective hydrolysis of aptamer I by **5**. Lane 1: alkaline hydrolysis; lane 2: incubation with **5** for 1 h; lane 3: control; lane 4: hydrolysis by RNAse T1; lane 5: control.*

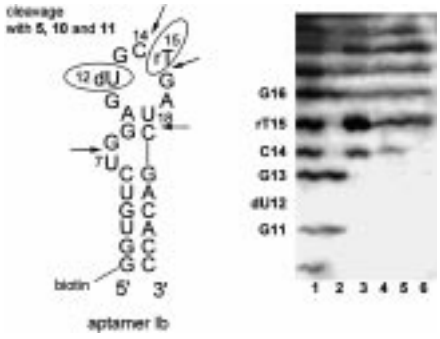

Figure 6. *PAGE result after sequence-selective hydrolysis of aptamer Ib by **5**, **10**, and **11**. Lane 1: alkaline hydrolysis; lane 2: hydrolysis by RNAse T1; lane 3: incubation with **5** for 1 h; lane 4: incubation with **11** (16.7 mM); lane 5: incubation with **11** (167 mM); lane 6: incubation with **10** (16.7 mM).*

11. This aptamer contains the modified bases deoxyuridine 12 (dU 12) and ribothymidine 15 (rT 15); in aptamer I these residues were uridines. The alkaline hydrolysis (Figure 6; lane 1) shows no spot at position 12, consistent with the absence of the 2′-hydroxy group at this residue. In lane 3 (reaction with **5**) the previously observed hydrolysis at positions 14 and 15 appeared again, but no hydrolysis at dU 12 was observed. This strongly supports the assumption that transesterification is the mode of action for the cleavage and makes radical or oxidative processes very unlikely. In lanes 4 – 6 the results of hydrolysis experiments with phenylalanine analogues, albeit in higher concentrations, are shown. It can be seen that in these cleavage reactions the same spots (C 14, rT 15) appear as with **5**, but with significantly lower intensity at 10- or 100-fold concentrations, respectively. These findings not only confirm the hydrolytic mechanism but also support the importance of the tyrosine hydroxy group for the cleavage.

Since we observed uridine-selective cleavage of aptamer I we were surprised that cleavage experiments performed with aptamer II showed only one band at U 23 (Figure 7). This seemed to contradict our hypothesis of selective cleavage at unpaired

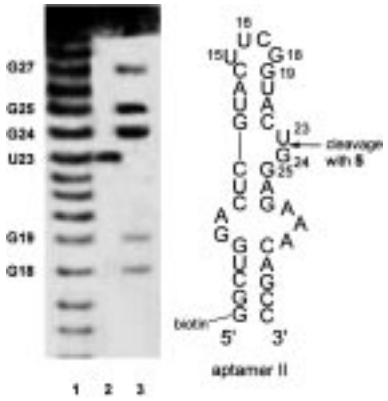

Figure 7. *PAGE result after sequence-selective hydrolysis of aptamer II by* ***5****. Lane 1: alkaline hydrolysis; lane 2: incubation with* ***5*** *for 1 h; lane 3: hydrolysis by RNAse T1.*

uridine residues. We therefore analyzed the tertiary structure of these aptamers, especially for noncanonical base pairs and small bond angles around the scissile phosphodiester bonds.[7] For aptamer II we found that residue U 15, at which no cleavage by **5** ocurred, is involved in a noncanonical base pair and can therefore not be regarded as an unpaired residue. In the other case where we observed no cleavage (aptamer II, U 16), the angle[8] around the phosphodiester bond was exceptionally small (92.4°; Figure 8).

This small angle disfavors the otherwise very efficient in- line mechanism for the cleavage of phosphodiester bonds. The angles for the other uridine residues in the loop region of aptamer I, for which cleavage with **5** was observed, were 115° and 146°. On the other hand, even with angles at purine residues in that range (125.7° for G 24 in aptamer II) no cleavage was observed. In this respect we want to point out that the data we used for the analysis of the aptamer structures were derived from aptamer – peptide complexes and not from the uncomplexed RNAs. Additionally, binding of **5** can alter the RNA conformation and may lead to different structures than the ones reported. Even if the present amount of data is not sufficient to propose the exact mode of action for the observed cleavage, the figures are such that we never observed cleavage at purine residues and only one minor spot for the cleavage at C 15 (aptamer I). Additionally, we found that no cleavage was observed for phosphodiester bonds with small bond angles. This disfavors the in-line mechanism, an effect that was already observed by others and described by Egli et al.[9] in the analysis of the different extent of hydrolysis due to metal- or spermine-induced conformational change in RNA. Our work adds another important aspect to the field of selective RNA cleavage with basic amines.[10] We have described a small molecule that can cleave RNA at unpaired uridines and therefore may become a useful tool for molecular biology. Additionally, deconvolution of these results may lead to new structural probes that can be used for the analysis of RNA as well as to new therapeutic approaches that make use of specific interactions of small molecules with RNA.

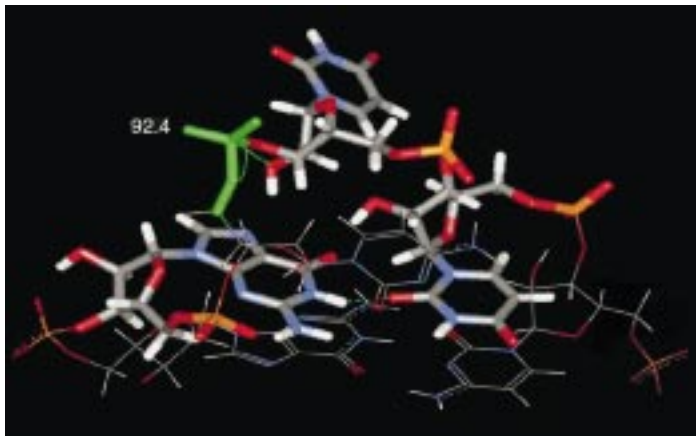

Figure 8. *Loop region of aptamer II. The noncanonical base pair G·U is shown as a stick representation. The angle between the 2′-hydroxy group of uridine 16, the connecting phosphorus atom, and the 5′-oxygen atom at C 17 (92.4°) is highlighted in green. For reasons of clarity, the bases other than U 15, U 16, and G 18 are depicted as wireframe models only.*

Experimental Section

The RNAs were purchased from Genset and were labeled with biotin at their 5′ ends. All experiments were performed in autoclaved Eppendorf reaction vessels. Extreme precaution was taken to avoid RNAse contamination. Water (Millipore quality) and all equipment had been treated with diethyl pyrocarbonate (DEPC) and then autoclaved prior to use. Control experiments in the presence of EDTA showed that the observed hydrolysis was not catalyzed by metal ion contaminations. To show that these results were no artifacts all experiments were reproduced with newly synthesized peptides. The RNA cleavage reaction was carried out at in a buffered solution (pH 7.4, 20 mM Tris-HCl) containing RNA (135 nM), peptides (167 μM), tyrosine derivatives (1.67 mM), and NaCl (20 mM). The reaction mixture was incubated for 2 h at room temperature unless otherwise stated. After the reaction the mixture was loaded onto a 20% (w/v)

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denaturing polyacrylamide gel. After electrophoresis the RNA was transferred onto a positively charged nylon membrane (Ambion) by electroblotting. After immobilization at 80 °C for 30 min, followed by the wash protocol described by the manufacturer, the RNA was visualized with streptavidin/alkaline phosphatase and CDP-star reagent (disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate) on Kodak film.

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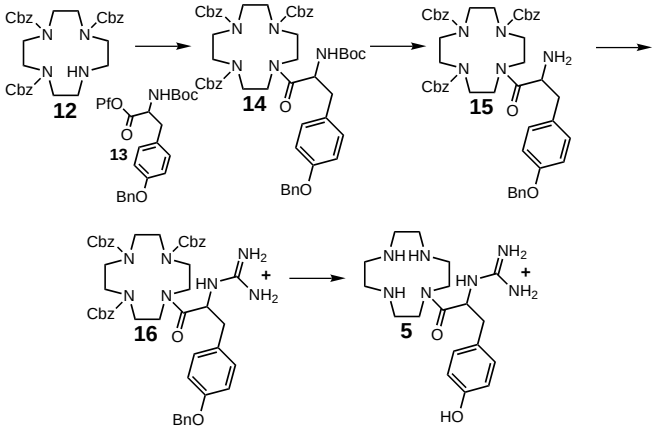
[5] Protected cyclen **12** was synthesized according to the method of Prasad et al.: J. S. Prasad, F. J. Okuniewics, E. J. Delaney, D. D. Dischino, *J. Chem. Soc. Perkin Trans. 1* **1991**, 3329. Synthesis of **5**: Anhydrous reactions were performed under argon in flame-dried glassware. Synthesis of **14**: Compound **12** (1.14 g, 1.99 mmol) dissolved in 5.5 mL DMF was stirred at room temperature. HOBT (244 mg, 1.81 mmol) and ester **13** (971 mg, 1.81 mmol) were added to this solution (HOBT = 1-hydroxy-1*H*-benzotriazole). After two days the reaction was quenched by adding aqueous $NaHCO_3$ and the aqueous layer was extracted with EtOAc (3 × 150 mL). The organic layer was washed with water and dried over Na_2SO_4. After evaporation the residue was purified by column chromatography on silica gel (EtOAc). Compound **14** (1.513 g, 90%) was isolated as a white solid. ^{13}C NMR: (100 MHz, $CDCl_3$, TMS): δ = 28.3, 39.25, 49.24, 49.54, 49.88, 50.84, 51.65, 60.36, 67.35, 69.88, 79.42, 114.71, 127.45, 127.92, 128.33, 128.37, 128.54, 128.70, 130.39, 136.07, 136.5, 137.0, 154.69, 156.13, 157.44, 157.70; ^{1}H NMR: (400 MHz, $CDCl_3$, TMS): δ = 1.45 (s, 9H), 2.68–3.80 (m, 18H), 4.60–4.74 (m, 1H), 4.90–5.15 (m, 8H), 6.78 (d, 2H, J = 8.0 Hz), 7.15 (d, 2H, J = 8.0 Hz), 7.24–7.51 (m, 20H); IR ($CHCl_3$): $\tilde{\nu}$ = 3434, 2999, 1699, 1644, 1473, 1421, 1368, 1245, 1170, 1026 cm^{-1}; MS (FAB): *m/z* (%): 929 (55) $[M]^+$, 828 (100), 575 (27), 441 (10), 307 (11), 154 (48). Synthesis of **15**: Deprotection of the *tert*-butoxycarbonyl (Boc) group was achieved following the method of R. A. Houghten et al.: R. A. Houghten, A. Beckman, J. M. Ostresh, *Int. J. Pept. Protein Res.* **1986**, *27*, 653. Compound **14** (589 mg, 0.64 mmol) was dissolved in 10 mL dioxane. Then conc H_2SO_4 (264 μL) was added. The reaction was quenched after 5 h with aqueous sat. K_2CO_3 to adjust the pH value to 11–12. The product was extracted with EtOAc and dried over Na_2SO_4. After evaporation the residue was purified by column chromatography on silica gel (EtOAc/MeOH, 1:1) to yield **15** (525 mg, 100%) as a white solid. ^{13}C NMR: (100 MHz, $CDCl_3$, TMS): δ = 49.55, 49.68, 49.70, 50.23, 67.05, 67.10, 67.42, 69.92, 114.89, 127.45, 127.88, 128.28, 128.39, 128.58, 130.61, 136.17, 136.38, 137.01, 157.78; ^{1}H NMR: (400 MHz, $CDCl_3$, TMS): δ = 2.46–3.65 (m, 19H), 4.80–5.20 (m, 8H), 6.71–7.14 (m, 4H), 7.17–7.44 (m, 20H); IR ($CHCl_3$): $\tilde{\nu}$ = 2999, 2964, 1697, 1473, 1420, 1367, 1245, 1121, 1027 cm^{-1}; MS (FAB): *m/z* (%): 828 (100) $[M]^+$, 630 (13), 575 (15), 307 (40), 154 (100). Synthesis of **16**: Ethyl diisopropylamine (84 μL, 0.6 mmol) was added to 3.5-dimethylpyrazole-1-carboxamidine nitrate (121 mg, 0.6 mmol) at room temperature. Then the free amine **15** (496 mg, 0.6 mmol) was dissolved in 833 μL DMF and added to the guanidinium reagent. After two days the product was precipitated as a white solid by adding diethyl ether. The product was then washed twice with diethyl ether. The residue was purified by column chromatography on silica gel (EtOAc/MeOH, 2:1). Compound **16** (333 mg, 64%) was isolated as a white solid. ^{13}C NMR: (100 MHz, $CDCl_3$ + CD_3OD, TMS): δ = 42.63, 49.0–50.53, 54.45, 67.60, 69.90, 114.90, 127.51, 128.48, 128.54, 128.63, 130.75, 135.84, 136.16, 136.90, 157.67, 158.03, 170.33; ^{1}H NMR: (400 MHz, $CDCl_3$, TMS): δ = 2.40–3.90 (m, 19H), 4.50–5.20 (m, 8H), 6.82 (d, 2H, J = 8.0 Hz), 6.07 (d, 2H, J = 8.0 Hz), 7.12–7.50 (m, 20H); IR ($CHCl_3$): $\tilde{\nu}$ = 2999, 2964, 1697, 1511, 1473, 1420, 1367, 1245, 1121, 1027 cm^{-1}; MS (FAB): *m/z* (%): 870 (80) $[M]^+$, 828 (24), 575 (13), 307 (44), 154 (100). Synthesis of **5** (Scheme 3): Compound **16** (328 mg, 0.38 mmol) was dissolved in 8.3 mL MeOH and 10% Pd/C (31 mg) was added. The reaction mixture was then hydrogenated for two days. After filtration the solvent was evaporated under reduced pressure. The product was dissolved in water and purified on IRA 400 and by reversed-phase chromatography (Waters Sep-Pak C18 cartridges, eluent: water/MeOH, 1:1). Compound **5** (129 mg, 91%) was isolated as a white solid. ^{13}C NMR: (100 MHz, D_2O, sodium 3-trimethylsilyl-[D_4]propionate ([D_4]TSP)): δ = 46.11, 46.74, 47.80, 49.62, 51.95, 55.02, 118.28, 130.20, 133.54, 157.32, 157.66, 179.68; ^{1}H NMR: (400 MHz, D_2O, [D_4]TSP): δ = 2.7–3.5 (m, 19H), 6.78–6.91 (m, 2H), 7.02–7.2 (m, 2H); IR ($CHCl_3$): $\tilde{\nu}$ = 3688, 3606, 3043, 2359, 1602, 1419, 1230, 930 cm^{-1}; MS (MALDI-TOF; Kompakt Maldi 3 from Kratos, α-cyanocinnamic acid): *m/z*: 377.6 which corresponds to $[M]^+$ of **5**.



Scheme 3. *Synthesis of **5**. Cbz = benzyloxycarbonyl; Pf = pentafluorophenyl.*

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[8] Angle spanned between the 2′-hydroxy group of U16, the phosphorus atom between U16 and C17, and the 5′-leaving group on C17.

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