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A Bis(guanidinium)alcohol Attached to a Hairpin Polyamide: Synthesis, DNA Binding, and Plasmid Cleavage

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Bis(guanidinium)alcohols have been designed to react with phosphodiester substrates in a fast transphosphorylation step, a quasi-intramolecular process taking place in contact ion pairs. Here the attachment of such compounds to Dervan-type hairpin polyamides is described. The resulting conjugate **1** binds to AT-rich DNA duplexes with affinity similar to that of the parent polyamide as shown by UV melting experiments and CD titrations. Conjugate **1** nicks plasmid DNA at concentrations ranging from micromolar to high nanomolar.

Introduction

The phosphodiester bonds in DNA are extremely stable against nucleophilic displacement and exhibit half-lives in the range of millions of years.[1] Therefore they represent an ideal linker element for building up and keeping genetic information. The covalent manipulation of DNA, on the other hand, is a big challenge in which artificial systems can rarely compete with natural enzymes. Most prominent for genome engineering is the CRISPR/Cas system, in which Cas9 acts as a programmable DNA endonuclease, guided by a natural or synthetic RNA strand.[2] Substrate recognition is in this case achieved through base pairing.

The construction of synthetic restriction enzymes generally requires a sequence recognition element attached to a DNA-cleaving unit.[3] Specific binding to double-stranded DNA can be achieved by protein domains,[4] triple-helix-forming oligonucleotides,[5] displacement loops,[6] or pyrrole-imidazole polyamides.[7] The last type of DNA ligand is based on *N*-methylpyrrole, *N*-methylimidazole, and γ-aminobutyric acid (GABA). With the GABA linker the polyamide can fold back as an antiparallel hairpin that binds DNA duplexes in the minor grove. With the aid of different combinations of *N*-methylpyrrole and *N*-methylimidazole, specific DNA duplexes rich in AT base pairs can be recognized. Such compounds are preferentially prepared on solid support,[7a] although liquid-phase synthesis can be advantageous when larger amounts of product are required.[8] In recent years polyamides have found their way into a wide range of applications, such as inhibition of transcription[9] or sequence-specific alkylation[10] of DNA and also DNA cleavage.[11]

The most prominent classes of artificial DNA cleavers are *zinc finger nucleases* and *transcription-activator-like effector nucleases* (TALENs).[4] Both use the natural FokI domain as active catalyst. Entirely synthetic systems are mostly based on metal ion complexes, in particular those of lanthanides.[3,12] DNA can also be cleaved by radicals attacking the carbohydrate framework. In this approach redox-active metal complexes such as EDTA/Fe^{2+} are used in the presence of oxygen and a reducing agent.[13]

Our group has previously shown that anion receptor molecules based on bis(guanidinium)alcohols can undergo fast phosphoryl transfer reactions in the absence of metal ions and are able to cleave plasmid DNA under physiological conditions.[14] Here we investigate the conjugate **1** (Scheme 1) of such a compound with a Dervan-type polyamide which was prepared by liquid-phase synthesis.

Results and Discussion

Synthesis

Conjugate **1** is based on the bis(guanidinium)alcohol unit (precursor: **17**, Scheme 4, below) and the ImPy-polyamide **16**, connected through an amide bond. The two parts were synthesized separately. Compound **17** was prepared as published previously.[14b] Compound **16** was retrosynthetically divided into three parts: the two trimers **8b** and **10b** (Scheme 2) plus the spacer **14** (Scheme 3).

Polyamide **16** was prepared by liquid-phase synthesis. The trimers **8b** and **10b** were assembled first, starting from monomers **2**–**5**.[15,16] Coupling of GABA ester **3** and the activated Py derivate **2** led to **6** in 91% yield. Hydrogenation at 40 bar (Pd/C) then reduced the nitro group. The crude product could be coupled with **2**, and O_2N-PyPy-GABA-OMe (**7**) precipitated from the reaction mixture (80%). Compound **7** was hydrogenated again and formed trimer **8a** on treatment with imidazole **5** (83%). Ester hydrolysis finally led to **8b** (93%).

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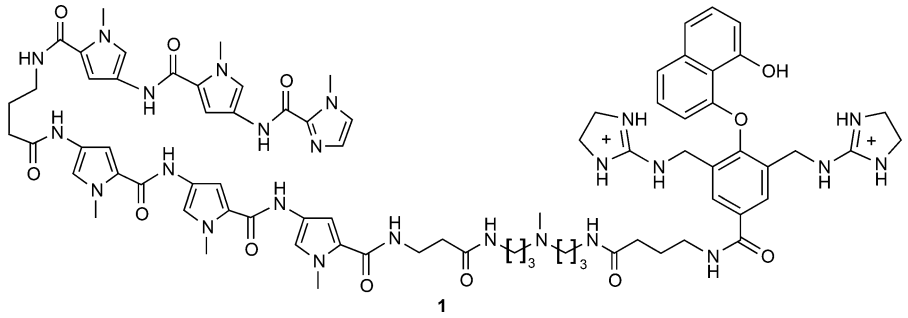
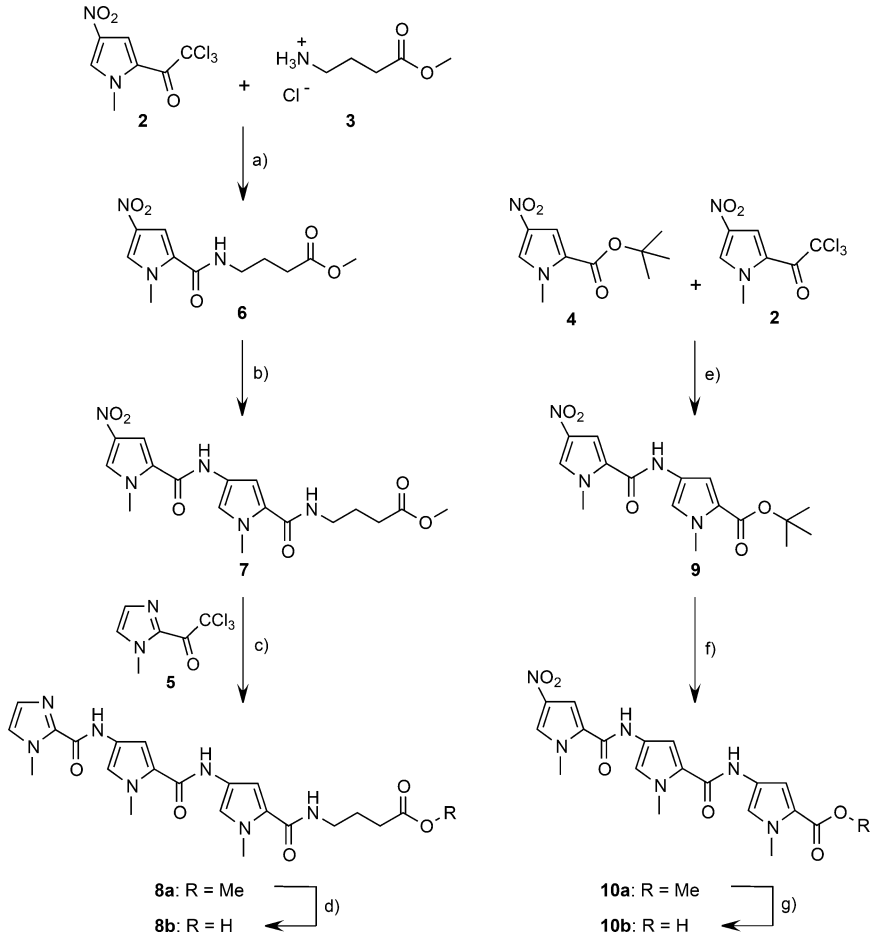


Scheme 1. Structure of the DNA cleaving conjugate **1** (counter ion: chloride).

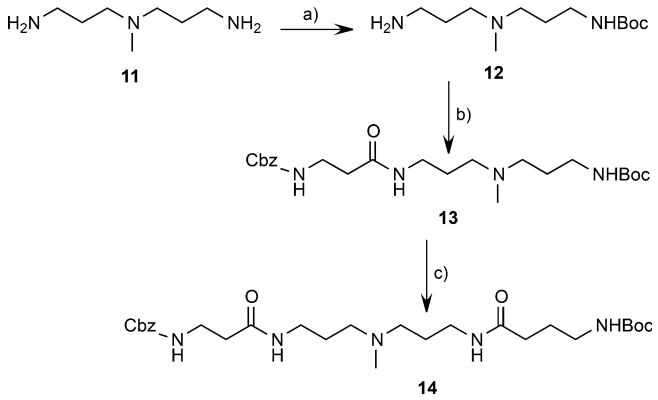


Scheme 2. a) DIEA, CH_2Cl_2, **2**, **3**, 16 h, RT, 91%; b) i. Pd/C, MeOH, 2 h, 40 bar H_2, 55 °C; ii. NaH, DIEA, CH_2Cl_2, **2**, 16 h, RT, 80%; c) i. Pd/C, MeOH, 3 h, 40 bar H_2, 60 °C; ii. DIEA, CH_2Cl_2, **5**, 16 h, RT, 83%; d) LiOH·H_2O, MeOH/H_2O, 16 h, 0 °C→RT, 93%; e) i. **4**, Pd/C, EtOAc, 3 h, 40 bar H_2, 55 °C; ii. DIEA, CH_2Cl_2, **2**, 16 h, RT, 85%; f) i. Pd/C, EtOAc, 3 h, 40 bar H_2, 55 °C; ii. DIEA, CH_2Cl_2, **2**, 16 h, RT, 75%; g) $TiCl_4$, CH_2Cl_2, 90 min, 0 °C→RT, 81%.

In a similar way monomer **4** was reduced to the corresponding amine and acylated with compound **2**. O_2N-PyPy-O*t*Bu **9** precipitated and was isolated by filtration (85%). Further reduction and acylation with **2** converted **9** into trimer **10a** (75%). Ester hydrolysis was achieved with $TiCl_4$ and yielded acid **10b** (81%).

The starting material for the synthesis of **14** was compound **11**, which was first treated with Boc anhydride to afford **12** in 79% yield (based on Boc_2O) after distillation. The residual amino group of **12** was then allowed to react with Cbz-β-Ala-OH to give **13** (87%). Partial deprotection with HCl followed by condensation with Boc-GABA produced linker **14** (87%).

Assembly of the three building blocks (Scheme 4) began with removal of Cbz from linker **14** and coupling with acid **10b**. The small excess of **10b** was removed by column chromatography to allow the isolation of **15** in 72% yield.



Scheme 3. a) Boc_2O, 1,4-dioxane, RT, overnight, 79%; b) Cbz-β-Ala, DIEA, HOBt, DIC, DMF, 60 °C, 4 h, 87%; c) i. AcCl, MeOH, quant.; ii. Boc-GABA, DIEA, HOBt, DIC, DMF, 80 °C, 4 h, 87%.

Then, the nitro group of **15** was reduced, and condensation with **8b** completed the synthesis of the hairpin amide (43%, higher yields are accessible in this step). Removal of Boc and condensation with acid **17** produced compound **18** (62%). After a final deprotection step, treatment with 4,5-dihydroimidazole-2-sulfonic acid[17] led to the target compound **1** as a yellowish foam after HPLC purification (19%).

Binding to DNA duplexes

Hairpin polyamides such as **19** are known to bind double-stranded DNA rich in AT base pairs in the minor groove and to effect considerable duplex stabilization. The duplex d(CATTGTTAGAC)$^{3'}$:d(GTCTAACAATG)$^{3'}$, for example, has a reported T_m value of (42.7 ± 0.3) °C that rises to (53.7 ± 0.7) °C upon addition of one equivalent of polyamide **19**.[18] To see if the presence of the bis(guanidinium) unit might alter the DNA affinity we repeated the melting experiment under identical conditions (see the Experimental Section and the Supporting Information) in the absence (42.0 ± 0.2) °C and in the presence (53.6 ± 1.5) °C of conjugate **1**. The conformity of the results shows that the two compounds **1** and **19** must have similar DNA affinities.

Hairpin polyamides **1**, **16**, and **19** are all achiral and therefore do not show any Cotton effect. However, upon binding to DNA the chiral environment is expected to induce CD bands in the region around 330 nm in which the UV absorption of the polyamides is not overlain by the absorption bands of DNA. Such induced CD bands are sensitive to local conformations. CD spectra of conjugate **1** bound to d(CATTGTTAGAC)$^{3'}$:d(GTCTAACAATG)$^{3'}$ (Supporting Information) look very similar to analogous spectra reported for compound **19**.[18] The bis(guanidinium) alcohol present in **1** apparently does not disturb the structure of the polyamide·DNA complex. When DNA is titrated with polyamides **1** or **19**, the intensities of induced CD bands as a function of ligand concentration also give access to binding constants. A value of $(7.3 \pm 1.3) \times 10^6\ M^{-1}$ has been reported for **19** and the 11-mer DNA duplex mentioned above.[18] Analogous titrations with conjugate **1** show a slightly reduced value of K_a ($(1.5 \pm 0.6) \times 10^6\ M^{-1}$, see the Supporting Information) in spite of the presence of two extra guanidinium ions.

DNA cleavage

For all cleavage studies the supercoiled plasmid pUC19 was used in 50 mM HEPES·NaOH buffer and analyzed by agarose gel electrophoresis. This technique separates and quantifies the supercoiled (form I), the nicked (form II), and the ring-opened form of the plasmid. pUC19 consists of 5372 nucleotides. Cleavage of any of the phosphodiester bonds and fragmentation of any of the nucleotides will convert supercoiled DNA into nicked DNA. The assay thus integrates thousands of different reaction channels. This fact and the strain of the supercoiled structure make plasmids quite sensitive to hydrolysis—in contrast to the exceptional stability of individual phosphodiester bonds.

Nonconjugated bis(guanidinium)alcohols such as **20** must be present in high-millimolar concentrations to nick pUC19 effectively.[14b] In the first set of experiments the conversion of supercoiled DNA into nicked DNA was determined as a function of cleaver concentration (Figure 1): 30–40% conversion was

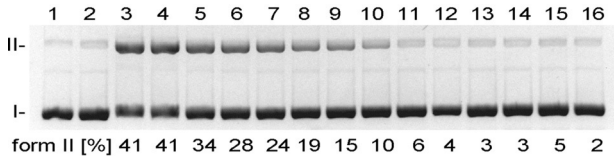
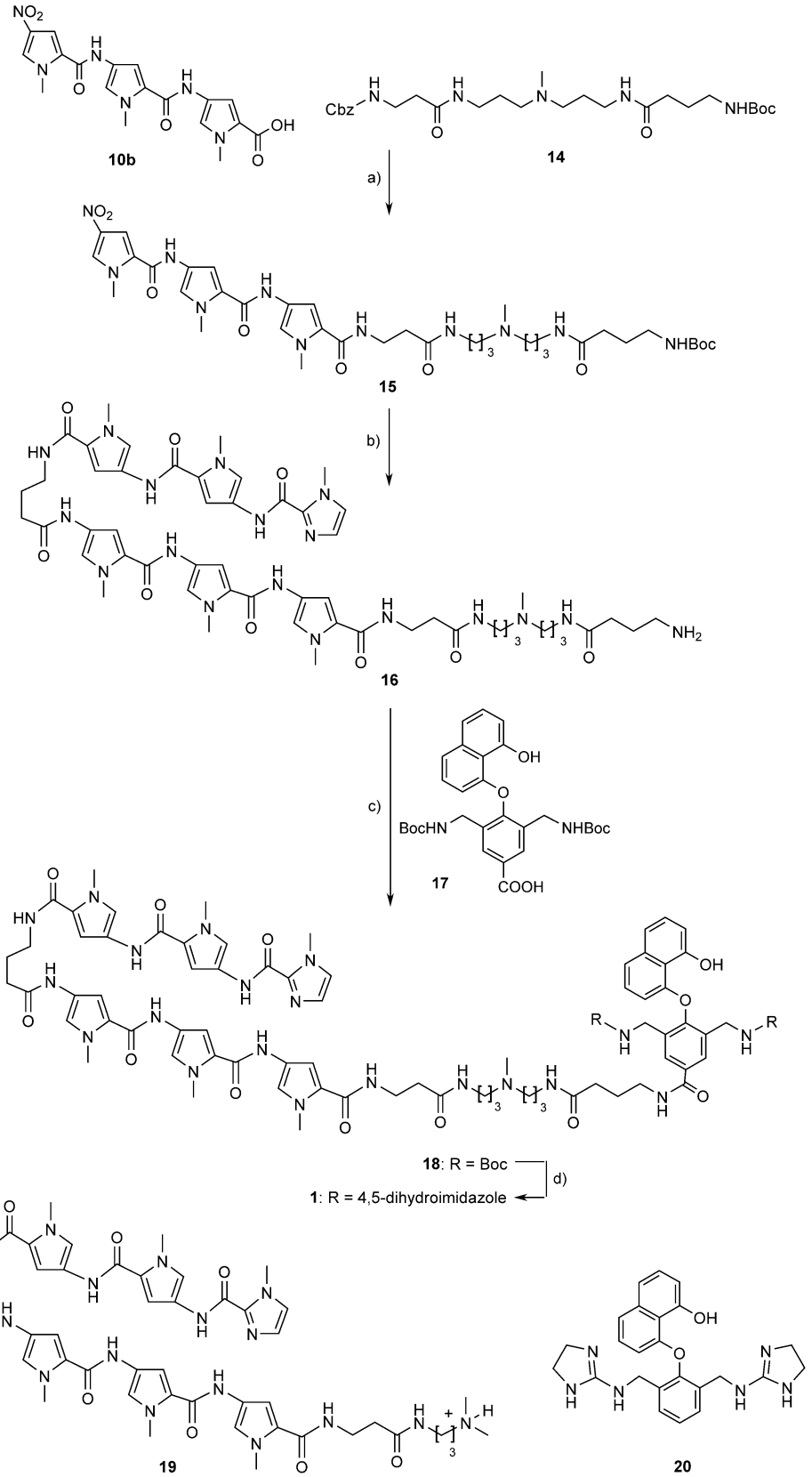


Figure 1. Concentration dependence of pUC19 DNA cleavage by **1**. Conditions: 22 nM DNA, 50 mM HEPES·NaOH (pH 7.0), 37 °C, 20 h; electrophoresis on 1% agarose gel, ethidium staining; inverted grayscale. I: plasmid-DNA form I. II: nicked circular DNA form II. Lane 1: unincubated control. Lanes 2–16: 0, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02, 0.01, 0.005, 0.002, and 0.001 μM, respectively (see the Supporting Information for a full-size picture of the gel).

achieved by conjugate **1** at concentrations 2000 times lower than those required for the unconjugated analogue **20** to give comparable effects. No linear DNA was observed under such conditions. Cleavage effects above background are seen at conjugate concentrations as low as 78 nM, at which no more than three or four molecules of compound **1** are present per copy of plasmid. Plasmid cleavage by unconjugated bis(guanidinium)alcohols becomes more effective with increasing pH values.[14b] In contrast, conjugate **1**, when tested between pH 7 and 9, works best in more acidic buffers (Figure 2).

Basic conditions could deprotonate the charged amino group of **1**. However, only a minor reduction in DNA affinity is observed upon removal of the terminal amine from compound **19**.[19] Thus, linker deprotonation cannot fully explain the change in reactivity.

Finally, the time course of plasmid cleavage was determined (Figure 3). After 8 h, conjugate **1** had converted nearly 30% of the plasmid into the nicked form, not much less than the 40% obtained after 20 h. The reason why the reaction stalls or goes into saturation is presently not known.



Scheme 4. a) i. **14**, Pd/C, MeOH, 40 bar H_2, 55 °C, 3 h; ii. **10b**, DIEA, HOBt, DIC, DMF, 60 °C, 4 h, 72%; b) i. Pd/C, MeOH, 40 bar H_2, 50 °C, 2 h; ii. **8b**, DIEA, HOBt, DIC, DMF, 60 °C, 3 h; iii. AcCl, MeOH, 43%; c) **17**, DIEA, HOBt, DIC, DMF, 60 °C, 3 h, 62%; d) i. AcCl, MeOH; ii. 2:1 MeOH/NEt_3, 4,5-dihydroimidazole-2-sulfonic acid, RT, 16 h, 19%.

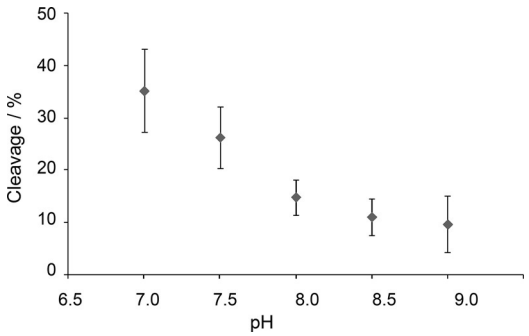


Figure 2. Plasmid cleavage as a function of pH: 22 nM pUC19 DNA, 10 μM conjugate **1**, 50 mM HEPES·NaOH, pH as given above, 37 °C, 20 h. Plasmid DNA is unstable in HEPES buffers below pH 7 (Figure S7). Mean values of three experiments. (Gel is shown in the Supporting Information.)

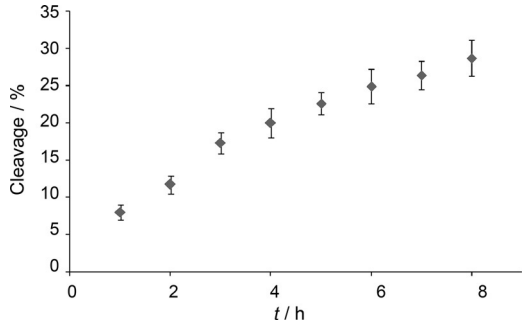


Figure 3. Plasmid cleavage as a function of time: 22 nM pUC19 DNA, 10 μM conjugate **1**, 50 mM HEPES·NaOH pH 7.0, 37 °C, reaction time as given above. Mean values of three experiments. (Gel is shown in the Supporting Information.)

Conclusions

Bis(guanidinium)alcohols have been designed to bind anionic phosphodiesters as contact ion pairs and to undergo fast phosphoryl transfer in which their hydroxy groups act as nucleophiles. In reactions with activated model substrates the resulting phosphorylation products can be isolated and fully characterized.[14] The naphthol OH group present in compounds **1** and **20** is also a good leaving group, which makes the phosphorylation step potentially reversible. In a formal sense bis(guanidinium)alcohols can be seen as models of type I DNA topoisomerases, enzymes that reversibly cleave and ligate DNA strands without consumption of ATP. They rely on tyrosine residues as nucleophiles and leaving groups in combination with arginine residues used for substrate binding and activation.[20] Reversible phosphorylation of tyrosine is also the mechanism of many recombinases and integrases.[21] In contrast to compound **20**, which requires millimolar concentrations for DNA cleavage, the polyamide conjugate **1** works down to the nanomolar range. In analogy with the reactions between **20** and model substrates (e.g., bis-(4-nitrophenyl)-phosphate), the products formed by **1** and DNA might be mixed phosphodiesters of its naphthol hydroxy group with oligonucleotide 5′- or 3′-phosphates. However, the mechanism is not yet confirmed (for unsuccessful studies, see the Supporting Information). We cannot rule out the possibility that such intermediates might undergo a fast religation step, thus making isolation of covalent intermediates a very difficult task. DNA cleavage induced by naphthoxy radicals is another mechanistic option. It is not excluded but seems less probable because DNA cleavage by **20** has been shown not to depend on oxygen.[14b] Finally, the hydroxy groups of **1** and **20** might act as general acids to destroy DNA by depurination. Further experiments to localize the cleavage sites of **1** are underway. A purine-specific cleavage pattern, for example, would point to an acidolytic mechanism whereas phosphoryl transfer would be expected to form covalent intermediates and not to show a preference for purines. The observation that **20** cleaves pUC19 more rapidly at higher pH, however, argues against acidolytic depurination. Thus, at present a transphosphorylation mechanism looks probable for conjugate **1**. However, further studies are required to see whether **1** cleaves DNA in a reversible way or even behaves as an artificial DNA topoisomerase.

Experimental Section

General: Flash column chromatography was performed with silica gel 60 (0.032–0.063 mm) purchased from Macherey–Nagel or Roth. Thin-layer chromatography (TLC) was performed with Merck 60 F_{254} silica gel plates (0.2 mm layer) with visualization under UV radiation (254 nm) or by staining with an acidic aqueous ammonium molybdate(IV) or ninhydrin solution; ^{1}H NMR (300 K): Bruker AM 250 operating at 250 MHz or Bruker AM 300 operating at 300 MHz. Chemical shifts in ppm. The solvent peaks were calibrated to 7.25 ppm ($CDCl_3$) or 2.50 ppm ([D_6]DMSO). ^{13}C NMR (complete proton decoupling): Bruker AM 250 operating at 60 MHz or Bruker AM 300 operating at 75 MHz. The solvent peaks were calibrated to 77.2 ppm ($CDCl_3$) or 39.4 ppm ([D_6]DMSO). COSY, HSQC, HMBC, and DEPT experiments were used to support the NMR assignment. Mass spectra were recorded with a Fisons VG Platform II (ESI) or a Fisons VG TOFSpec (MALDI). FTIR: PerkinElmer Spectrum Two. Melting points are not corrected. HPLC: Pump Waters 590 and detector Waters 440. DIC = *N*,*N*-diisopropylcarbodiimide, DIEA = diisopropylethylamine, Hex = *n*-hexane, HOBt = 1-hydroxybenzotriazole (> 86 %).

Synthesis

O_2N-Py-GABA-OMe (***6***): DIEA (6.69 mL, 39.06 mmol) was added to a suspension of methyl γ-aminobutyrate·HCl (**3**,[16] 4.00 g, 26.04 mmol) in CH_2Cl_2 (35 mL). Subsequently, **2**[22] (7.78 g, 28.64 mmol) was added. After the system had been stirred for 16 h at room temperature, the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (Hex/EtOAc 1:2) to afford **6** as a colorless solid (6.36 g, 91 %). M.p. 120 °C; ^{1}H NMR (250 MHz, [D_6]DMSO): δ = 8.39 (t, J = 5.7 Hz, 1 H; N*H*), 8.11 (d, J = 2.0 Hz, 1 H; Py-*H*), 7.42 (d, J = 2.1 Hz, 1 H; Py-*H*) 3.90 (s, 3 H; N–CH_3), 3.58 (s, 3 H; COOCH_3), 3.21 (q, J = 6.8 Hz, 2 H; CH_2), 2.36 (t, J = 7.4 Hz, 2 H; CH_2), 1.75 ppm (quin, J = 7.1 Hz, 2 H; CH_2); ^{13}C NMR (60 MHz, [D_6]DMSO): δ = 173.0, 159.8, 133.7, 127.7, 126.4, 107.2, 51.2, 37.9, 37.2, 30.7, 24.3 ppm; IR: $\tilde{\nu}$ = 3391, 3131, 2945, 2885, 1731, 1662, 1543, 1520, 1506, 1485, 1465, 1450, 1427, 1408, 1353, 1312, 1280, 1197, 1172, 1132, 1100, 1053, 979, 893, 849, 810, 745, 708, 587, 521, 504, 458 cm^{-1}; MS (ESI$^+$): *m*/*z*: calcd for $C_{11}H_{15}N_3O_5 + H^+$: 270.1 $[M+H]^+$; found: 270.5; elemental analysis calcd (%) for $C_{11}H_{15}N_3O_5$: C 49.07, H 5.62, N 15.61; found: C 48.96, H 5.40, N 15.63.

*O_2N-PyPy-GABA-OMe (**7**)*: Compound **6** (2.00 g, 7.40 mmol) was added to a suspension of Pd/C (0.30 g, 10% on active charcoal) in anhydrous MeOH (30 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 2 h at 55 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated under reduced pressure. After drying of the product in vacuo, the product was dissolved in CH_2Cl_2 (10 mL). The resulting solution was dropped into a solution of **2** (2.40 g, 8.80 mmol) in CH_2Cl_2 (30 mL). After that NaH (60% in mineral oil, 0.29 g, 7.40 mmol) was added to the solution in portions over 30 min, followed by DIEA (1.91 mL, 11.14 mmol). During stirring for 16 h at room temperature a yellow solid precipitated. Water (20 mL) was carefully added to the suspension. The suspension was filtered, and the residue was washed with H_2O, CH_2Cl_2, and MeOH to yield **7** as a yellow solid (2.31 g, 80%). M.p. 200 °C; ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 10.24 (s, 1H; N*H*), 8.19 (d, J = 1.6 Hz, 1H; Py-*H*), 8.09 (t, J = 6.7 Hz, 1H; N*H*), 7.59 (d, J = 2.0 Hz, 1H; Py-*H*), 7.22 (d, J = 1.9 Hz, 1H; Py-*H*), 6.87 (d, J = 1.9 Hz, 1H; Py-*H*), 3.97 (s, 3H; N–C*H*$_3$), 3.82 (s, 3H; N–C*H*$_3$), 3.60 (s, 3H; COOC*H*$_3$), 3.20 (q, J = 6.7 Hz, 2H; NC*H*$_2$), 2.36 (t, J = 7.4 Hz, 2H; C*H*$_2$), 1.75 ppm (quin, J = 7.0 Hz, 2H; CH$_2$C*H*$_2$CH$_2$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ = 173.1, 161.2, 156.9, 133.8, 128.1, 126.3, 123.2, 121.3, 118.0, 107.5, 104.1, 51.2, 37.7, 37.4, 35.9, 30.8, 24.6 ppm; IR: $\tilde{\nu}$ = 3380, 3333, 3097, 2924, 2854, 1716, 1671, 1644, 1562, 1531, 1492, 1467, 1438, 1415, 1402, 1380, 1360, 1334, 1300, 1250, 1209, 1166, 1144, 1114, 1082, 1059, 1008, 985, 964, 890, 867, 807, 777, 757, 748, 717, 681, 660, 621, 590, 582, 490, 462 cm^{-1}; MS (ESI$^+$): *m/z*: calcd for $C_{17}H_{21}N_5O_6 + H^+$: 392.2; found: 392.2 [*M*+H]$^+$, 414.2 [*M*+Na]$^+$; HRMS (MALDI) *m/z:* calcd for $C_{17}H_{21}N_5O_6$: 391.14863 [*M*]; found: 391.1487.

*ImPyPy-GABA-OH (**8b**)*: Compound **7** (2.00 g, 5.11 mmol) was added to a suspension of Pd/C (0.30 g, 10% on active charcoal) in anhydrous MeOH (30 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 3 h at 60 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated. After drying of the product in vacuo, the product was dissolved in CH_2Cl_2 (10 mL). The resulting solution was dropped into a solution of **5** (1.39 g, 6.13 mmol) in CH_2Cl_2 (30 mL). DIEA (1.31 mL, 7.67 mmol) was added. After the system had been stirred for 16 h at room temperature, the solvent was evaporated, and the crude product was purified by column chromatography (Hex/EtOAc/CH_2Cl_2 2:9:1). The product was recrystallized from EtOH to yield **8a** as a colorless solid (1.98 g, 83%). M.p. 128–129 °C; ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 10.44 (s, 1H; N*H*), 9.91 (s, 1H; N*H*), 8.03 (t, J = 5.7 Hz, 1H; N*H*), 7.39 (d, J = 0.8 Hz, 1H; Im-*H*), 7.29 (d, J = 1.7 Hz, 1H; Py-*H*), 7.18 (d, J = 1.7 Hz, 1H; Py-*H*), 7.16 (d, J = 1.9 Hz, 1H; Py-*H*), 7.04 (d, J = 0.8 Hz, 1H; Im-*H*), 6.88 (d, J = 1.9 Hz, 1H; Py-*H*), 4.00 (s, 3H; N–C*H*$_3$), 3.85 (s, 3H; N–C*H*$_3$), 3.80 (s, 3H; N–C*H*$_3$), 3.59 (s, 3H; COOC*H*$_3$), 3.19 (q, J = 6.1 Hz, 2H; C*H*$_2$), 2.34 (t, J = 7.4 Hz, 2H; C*H*$_2$), 1.74 ppm (quin, J = 7.1 Hz, 2H; C*H*$_2$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ = 173.1, 161.3, 158.4, 156.1, 138.8, 127.0, 126.3, 123.0, 122.9, 122.0, 121.4, 118.6, 117.8, 104.9, 104.3, 51.2, 37.7, 36.0, 35.8, 35.0, 30.8, 24.6 ppm; IR: $\tilde{\nu}$ = 3407, 3267, 2951, 1716, 1671, 1632, 1578, 1542, 1519, 1467, 1450, 1431, 1403, 1380, 1273, 1255, 1202, 1179, 1145, 1124, 1065, 1010, 979, 937, 883, 813, 785, 764, 736, 699, 660, 639, 620, 548 cm^{-1}; MS (ESI$^+$): *m/z*: calcd for $C_{22}H_{27}N_7O_5 + H^+$: 470.2; found: 470.3 [*M*+H]$^+$, 492.3 [*M*+Na]$^+$; elemental analysis calcd (%) for $C_{22}H_{27}N_7O_5$: C 56.28, H 5.80, N 20.88; found: C 56.02, H 6.00, N 20.96.

Compound **8a** (1.00 g, 2.1 mmol) was suspended in a mixture of MeOH/H_2O (3:1, 40 mL) and cooled to 0 °C. LiOH·H_2O (0.44 g, 10.5 mmol) was added, and the mixture was stirred for 16 h. During this time the solution was allowed to warm up to room temperature. The solvent was evaporated, and the crude product was purified by column chromatography (CH_2Cl_2/MeOH 5:1) to yield **8b** as a colorless solid (0.90 g, 93%). M.p. 174 °C; ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 12.02 (br s, 1H; COO*H*), 10.41 (s, 1H; N*H*), 9.89 (s, 1H; N*H*), 8.02 (t, J = 5.5 Hz, 1H; N*H*), 7.38 (s, 1H; Im-*H*), 7.28 (d, J = 1.6 Hz, 1H; Py-*H*), 7.18 (d, J = 1.7 Hz, 1H; Py-*H*), 7.16 (d, J = 1.7 Hz, 1H; Py-*H*), 7.04 (s, 1H; Im-*H*), 6.88 (d, J = 1.7 Hz, 1H; Py-*H*), 4.00 (s, 3H; N–C*H*$_3$), 3.85 (s, 3H; N–C*H*$_3$), 3.80 (s, 3H; N–C*H*$_3$), 3.19 (q, J = 6.5 Hz, 2H; C*H*$_2$), 2.25 (t, J = 7.5 Hz, 2H; C*H*$_2$), 1.71 ppm (quint., J = 7.1 Hz, 2H; C*H*$_2$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ = 174.5, 161.3, 158.5, 156.1, 138.8, 127.0, 126.3, 123.1, 123.0, 122.1, 121.4, 118.6, 117.8, 104.9, 104.3, 37.9, 36.1, 35.9, 35.1, 31.4, 24.8 ppm; IR: $\tilde{\nu}$ = 3377, 3302, 2951, 1733, 1714, 1624, 1582, 1540, 1520, 1469, 1428, 1400, 1357, 1269, 1206, 1172, 1123, 1063, 1006, 935, 886, 821, 797, 776, 762, 696, 673, 635, 614 cm^{-1}; MS (ESI$^+$): *m/z*: calcd for $C_{21}H_{25}N_7O_5 + H^+$: 456.2; found: 456.3 [*M*+H]$^+$, 478.4 [*M*+Na]$^+$.

*O_2N-PyPy-OtBu (**9**)*: Compound **4**[15] (4.00 g, 17.68 mmol) was added to a suspension of Pd/C (0.40 g, 10% on active charcoal) in anhydrous EtOAc (30 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 3 h at 55 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated. After drying of the product in vacuo, the product was dissolved in CH_2Cl_2 (10 mL). The resulting solution was added dropwise to a solution of **2** (5.28 g, 19.45 mmol) in CH_2Cl_2 (30 mL), followed by DIEA (4.54 mL, 26.52 mmol). During stirring for 16 h at room temperature a yellow solid precipitated. The suspension was filtered, and the residue was washed with CH_2Cl_2 to yield **9** as a yellow solid (5.23 g, 85%). M.p. 217–218 °C; ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 10.20 (s, 1H; N*H*), 8.17 (d, J = 1.9 Hz, 1H; Py-*H*), 7.56 (d, J = 2.1 Hz, 1H; Py-*H*), 7.38 (d, J = 1.9 Hz, 1H; Py-*H*), 6.83 (d, J = 2.0 Hz, 1H; Py-*H*), 3.95 (s, 3H; N–C*H*$_3$), 3.81 (s, 3H; N–C*H*$_3$), 1.50 ppm (s, 9H; C(C*H*$_3$)$_3$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ = 159.8, 156.8, 133.8, 128.2, 126.0, 121.8, 120.4, 120.2, 108.3, 107.5, 79.7, 37.4, 36.2, 28.0 ppm; IR: $\tilde{\nu}$ = 3402, 1699, 1664, 1557, 1531, 1499, 1468, 1436, 1405, 1386, 1369, 1304, 1273, 1243, 1204, 1165, 1151, 1100, 1084, 1060, 843, 832, 812, 789, 748, 654, 633, 588, 528, 469 cm^{-1}; MS (ESI$^+$): *m/z*: calcd for $C_{16}H_{20}N_4O_5 + H^+$: 349.1; found: 349.3 [*M*+H]$^+$; elemental analysis calcd (%) for $C_{16}H_{20}N_4O_5$: C 55.17, H 5.79, N 16.08; found: C 55.13, H 5.88, N 16.25.

*O_2N-PyPyPy-OH (**10b**)*: Compound **9** (2.00 g, 5.74 mmol) was added to a suspension of Pd/C (0.20 g, 10% on active charcoal) in anhydrous EtOAc (30 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 3 h at 55 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated. After drying of the product in vacuo, the reduced **9** was dissolved in CH_2Cl_2 (10 mL). The resulting solution was added dropwise to a solution of **2** (1.72 g, 6.32 mmol) in CH_2Cl_2 (30 mL). DIEA (1.47 mL, 8.61 mmol) was added. After the system had been stirred for 16 h at room temperature, the solvent was evaporated, and the crude product was purified by column chromatography (Hex/EtOAc 1:1) to yield **10a** as a yellow solid (2.05 g, 75%). M.p. 178 °C; ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 10.28 (s, 1H; N*H*), 9.92 (s, 1H; N*H*), 8.19 (d, J = 2.0 Hz, 1H; Py-*H*), 7.59 (d, J = 2.0 Hz, 1H; Py-*H*), 7.40 (d, J = 2.0 Hz, 1H; Py-*H*), 7.25 (d, J = 1.7 Hz, 1H; Py-*H*), 7.07 (d, J = 1.9 Hz, 1H; Py-*H*), 6.85 (d, J = 2.0 Hz, 1H; Py-*H*), 3.97 (s, 3H; N–C*H*$_3$), 3.86 (s, 3H; N–C*H*$_3$), 3.81 (s, 3H; N–C*H*$_3$), 1.51 ppm (s, 9H; C(C*H*$_3$)$_3$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ = 159.9, 158.3, 156.9, 133.8, 128.2, 126.3, 122.8, 122.6, 121.5, 120.2, 120.1, 118.7, 108.4, 107.6, 104.6, 79.6, 37.4, 36.2, 36.1, 28.1 ppm; IR: $\tilde{\nu}$ = 3412, 3372, 3141, 2977, 1715, 1697, 1674, 1645, 1592, 1556, 1522, 1498, 1434, 1422, 1403, 1367, 1315, 1275, 1248, 1216, 1168, 1123, 1102, 1084, 1055, 1008,

987, 890, 848, 816, 777, 751, 742, 714, 668, 639, 600, 541 cm^{-1}; MS (ESI^+): *m/z*: calcd for $C_{22}H_{26}N_6O_6+H^+$: 471.2; found: 471.8 $[M+H]^+$, 493.9 $[M+Na]^+$; elemental analysis calcd (%) for $C_{22}H_{26}N_6O_6$: C 56.16, H 5.57, N 17.86; found: C 56.13, H 5.45, N 18.27.

Compound **10a** (2.00 g, 4.25 mmol) was diluted in anhydrous CH_2Cl_2 (40 mL). The solution was cooled to 0 °C, and a solution of $TiCl_4$ (0.93 mL, 8.50 mmol) in anhydrous CH_2Cl_2 (15 mL) was added dropwise. The solution was stirred for a further 30 min at 0 °C and was then allowed to warm to room temperature. HCl (1 N, 100 mL) was added slowly, and the solid precipitated. The solid was filtered, and the residue was washed with MeOH. After drying in vacuo **10b** was obtained as a yellow solid (1.43 g, 81%). M.p. 252 °C; ^1H NMR (250 MHz, [D$_6$]DMSO): $\delta=12.17$ (br s, 1H; COO*H*), 10.32 (s, 1H; N*H*), 9.96 (s, 1H; N*H*), 8.19 (d, $J=1.7$ Hz, 1H; Py-*H*), 7.61 (d, $J=2.0$ Hz, 1H; Py-*H*), 7.43 (d, $J=1.9$ Hz, 1H; Py-*H*), 7.27 (d, $J=1.6$ Hz, 1H; Py-*H*), 7.06 (d, $J=1.7$ Hz, 1H; Py-*H*), 6.85 (d, $J=1.7$ Hz, 1H; Py-*H*), 3.96 (s, 3H; N–CH_3), 3.86 (s, 3H; N–CH_3), 3.82 ppm (s, 3H; N–CH_3); ^{13}C NMR (60 MHz, [D$_6$]DMSO): $\delta=158.3$, 156.9, 133.8, 128.2, 126.3, 123.1, 122.2, 121.4, 118.5, 107.6, 107.5, 104.5, 37.4, 36.1, 36.0 ppm; IR: $\tilde{\nu}=3410$, 3379, 3138, 3110, 2924, 2853, 1674, 1645, 1591, 1560, 1522, 1498, 1435, 1420, 1402, 1355, 1309, 1257, 1210, 1174, 1153, 1123, 1110, 1062, 1005, 988, 889, 856, 834, 816, 779, 750, 738, 709, 666, 645, 591, 565, 520, 466 cm^{-1}; MS (ESI^-): *m/z*: calcd for $C_{18}H_{18}N_6O_6-H^+$: 413.1; found: 413.7 $[M-H]^+$.

*3-(*tert*-Butyloxycarbonylaminopropyl)-3′-aminopropyl*-N*-methylamine (**12**)*: Compound **11** (29.55 mL, 183 mmol) was dissolved in 1,4-dioxane (50 mL). A solution of Boc_2O (10.00 g, 45.8 mmol) in 1,4-dioxane (50 mL) was added dropwise to the resulting solution over 2 h. The colorless suspension was stirred overnight. Afterwards the solvent was evaporated; the residue was redissolved in water and extracted with CH_2Cl_2. The combined organic layer was washed with water and dried over $MgSO_4$, and the solvent was evaporated. The crude product was distilled in vacuo (1 mbar, 200 °C) to afford a colorless oil in 79% yield (8.84 g); ^1H NMR (250 MHz, $CDCl_3$): $\delta=5.47$ (br s, 1H; N*H*), 3.13 (q, $J=6.0$ Hz, 2H; NHCH_2), 2.70 (t, $J=6.9$ Hz, 2H; NH_2CH_2), 2.36–2.30 (m, 4H; 2CH_2), 2.14 (s, 3H; N–CH_3), 1.64–1.54 (m, 6H; CH_2N(Me)CH_2, NH_2), 1.39 ppm (s, 9H; C(CH_3)$_3$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): $\delta=155.5$, 77.0, 55.1, 54.9, 41.7, 40.0, 38.3, 30.9, 28.1, 27.2 ppm, IR: $\tilde{\nu}=3356$, 2974, 2933, 2866, 2793, 1693, 1517, 1454, 1390, 1364, 1273, 1249, 1168, 1072, 1047, 1002, 971, 861, 778, 643, 461 cm^{-1}; HRMS (MALDI): *m/z*: calcd for $C_{12}H_{27}N_3O_2+H^+$: 246.21760; found: 246.2178 $[M+H]^+$.

*Cbz-β-Ala-C_3-N(Me)-C_3-NHBoc (**13**)*: Compound **12** (3.00 g, 12.23 mmol), Cbz-β-Ala-OH (3.28 g, 14.67 mmol), DIEA (4.19 mL, 24.45 mmol), and HOBt (2.26 g, 14.67 mmol) were dissolved in anhydrous DMF (20 mL) and warmed to 60 °C. After that temperature had been reached, DIC (3.79 mL, 24.45 mmol) was added, and the brown solution was stirred for 4 h. The solution was allowed to cool to room temperature and was then slowly added dropwise to a solution of sat. $NaHCO_3/H_2O$ (1:4, 100 mL). The resulting yellow solution was extracted three times with CH_2Cl_2, and the combined organic layer was washed with water and dried ($MgSO_4$). The solvent was evaporated, and the crude product was purified by column chromatography (EtOAc/MeOH 6:1+1% NEt_3) to yield **13** as a colorless oil (4.81 g, 87%); ^1H NMR (250 MHz, $CDCl_3$): $\delta=7.26$–7.22 (m, 5H; benzyl-*H*), 7.12 (br s, 1H; N*H*), 5.67 (br s, 1H; N*H*), 5.11 (br s, 1H; N*H*), 5.00 (s, 2H; benzyl-CH_2), 3.39 (q, $J=6.1$ Hz, 2H; CH_2), 3.23 (q, $J=6.0$ Hz, 2H; CH_2), 3.08 (q, $J=5.5$ Hz, 2H; NHCH_2), 2.35–2.26 (m, 6H; $CH_2CH_2CH_2$), 2.09 (s, 3H; N–CH_3), 1.59–1.50 (m, 4H; CH_2N(Me)CH_2), 1.35 ppm (s, 9H; C(CH_3)$_3$); ^{13}C NMR (60 MHz, $CDCl_3$): $\delta=171.2$, 156.4, 156.1, 136.6, 128.3, 127.9, 127.8, 79.0, 66.4, 55.8, 55.5, 41.6, 38.9, 38.3, 37.3, 35.9, 28.3, 27.4, 26.0 ppm; IR: $\tilde{\nu}=3320$, 3064, 2941, 2798, 1690, 1649, 1526, 1454, 1391, 1365, 1342, 1246, 1167, 1138, 1055, 1002, 861, 777, 737, 697, 607, 576, 460 cm^{-1}, MS (ESI^+): *m/z*: calcd for $C_{23}H_{38}N_4O_5+H^+$: 451.3; found: 451.4 $[M+H]^+$, 473.2 $[M+Na]^+$; elemental analysis calcd (%) for $C_{23}H_{38}N_4O_5$: C 61.31, H 8.50, N 12.43; found: C 61.06, H 8.64, N 12.17.

*Cbz-β-Ala-C_3-N(Me)-C_3-GABA-NHBoc (**14**)*: Dry MeOH (5 mL) was cooled to 0 °C, and acetyl chloride (1 mL) was slowly added dropwise, followed by stirring for 30 min. Compound **13** (1.50 g, 3.33 mmol) was dissolved in anhydrous MeOH (5 mL) and added dropwise to the acidic MeOH solution, and the mixture was stirred at 0 °C for 30 min, followed by 60 min at ambient temperature. When the reaction was complete, the solvent was evaporated, and the residue was dried in vacuo. The deprotected **13**, Boc-GABA (0.81 g, 3.99 mmol), DIEA (2.28 mL, 13.32 mmol), and HOBt (0.62 g, 3.99 mmol) were dissolved in anhydrous DMF (10 mL) and warmed up to 60 °C. After that temperature had been reached, DIC (1.03 mL, 6.66 mmol) was added, and the resulting solution was stirred for 4 h at 60 °C. The solution was allowed cool to room temperature and was then slowly added dropwise to a solution of sat. $NaHCO_3/H_2O$ (1:4, 50 mL). The resulting yellow solution was extracted three times with CH_2Cl_2. The combined organic layer was washed with water and dried ($MgSO_4$). The solvent was evaporated, and the crude product was purified by column chromatography (EtOAc/MeOH 6:1+1% NEt_3) to yield **14** as a colorless oil (1.54 g, 87%); ^1H NMR (250 MHz, $CDCl_3$): $\delta=7.40$–7.30 (m, 5H; phenyl), 7.17 (br s, 1H; N*H*), 7.03 (br s, 1H; N*H*), 5.72 (br s, 1H; N*H*), 5.08 (s, 2H; benzyl-CH_2), 5.00 (br s, 1H; N*H*), 3.47 (q, $J=6.2$ Hz, 2H; CH_2), 3.30 (q, $J=6.1$ Hz, 4H; 2CH_2), 3.12 (q, $J=6.3$ Hz, 2H; CH_2), 2.46–2.38 (m, 6H; CH_2), 2.23–2.17 (m, 5H; CH_3 and CH_2), 1.77 (quin, $J=7.0$ Hz, 2H; CH_2), 1.66 (quin, $J=6.4$ Hz, 4H; CH_2), 1.42 ppm (s, 9H; C(CH_3)$_3$); ^{13}C NMR (60 MHz, $CDCl_3$): $\delta=172.8$, 171.4, 156.6, 156.5, 136.6, 128.4, 128.0, 127.9, 77.3, 66.5, 55.7, 55.6, 41.6, 39.9, 38.0, 37.4, 36.0, 33.8, 28.4, 26.7, 26.4 ppm. IR: $\tilde{\nu}=3297$, 3063, 2935, 2798, 1684, 1640, 1533, 1453, 1390, 1365, 1341, 1267, 1239, 1168, 1140, 1108, 1028, 1001, 910, 874, 851, 779, 750, 729, 697, 630, 618, 576, 465 cm^{-1}; HRMS (MALDI): *m/z*: calcd for $C_{27}H_{45}N_5O_6+H^+$: 536.34426; found: 536.3437 $[M+H]^+$.

*O_2N-PyPyPy-β-Ala-C_3-N(Me)-C_3-GABA-NHBoc (**15**)*: Compound **14** (1.00 g, 2.22 mmol) was added to a suspension of Pd/C (0.30 g, 10% on active charcoal) in anhydrous MeOH (20 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 3 h at 55 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated. After drying of the product in vacuo, the product was dissolved in anhydrous DMF. The resulting solution was added dropwise to a solution of **10b** (1.24 g, 2.99 mmol) in abs. DMF (10 mL), followed by DIEA (1.28 mL, 7.47 mmol) and HOBt (0.46 g, 2.99 mmol). The solution was heated to 60 °C, and DIC (0.77 mL, 4.98 mmol) was added. The solution was stirred for 4 h at 60 °C. Afterwards the solution was added to a mixture of sat. $NaHCO_3$/water (1:4, 50 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, and the solvent was evaporated. The crude product was purified by column chromatography (CH_2Cl_2/MeOH 9:1+1% NEt_3) to yield **15** as a yellow foam (1.28 g, 72%). ^1H NMR (250 MHz, [D$_6$]DMSO): $\delta=10.31$ (s, 1H; NH), 9.95 (s, 1H; NH), 8.18 (d, $J=1.6$ Hz, 1H; Py-*H*), 8.00 (t, $J=5.5$ Hz, 1H; NH), 7.90 (t, $J=5.5$ Hz, 1H; NH), 7.79 (t, $J=5.5$ Hz, 1H; NH), 7.61 (d, $J=2.1$ Hz, 1H; Py-*H*), 7.28 (d, $J=1.7$ Hz, 1H; Py-*H*), 7.20 (d, $J=1.6$ Hz, 1H; Py-*H*), 7.04 (d, $J=1.8$ Hz, 1H; Py-*H*), 6.84 (d, $J=1.7$ Hz, 1H; Py-*H*), 6.77 (t, $J=5.4$ Hz, 1H; N*H*), 3.97 (s, 3H; N–CH_3), 3.86 (s, 3H; N–CH_3), 3.81 (s, 3H; N–CH_3), 3.37 (q, $J=6.7$ Hz, 4H; CH_2), 3.08–3.05 (m, 4H; CH_2), 2.88 (q, $J=6.5$ Hz, 2H; CH_2), 2.36–2.28 (m, 4H; CH_2),

2.13 (s, 3H; N–CH_3), 2.03 (t, J=7.7 Hz, 2H; CH_2), 1.63–1.49 (m, 6H; CH_2), 1.36 ppm (s, 9H; $C(CH_3)_3$); ^{13}C NMR (60 MHz, [D$_6$]DMSO): δ= 171.6, 170.4, 161.2, 158.3, 156.9,155.5, 133.8, 128.1, 126.3, 123.0, 122.8, 122.1, 121.4, 118.6, 117.9, 107.6, 104.5, 104.1, 77.4, 54.6, 52.0, 48.5, 45.6, 41.4, 37.4, 36.7, 36.1, 35.9, 35.5, 35.4, 32.9, 28.2, 26.6, 25.8 ppm; IR: $\tilde{\nu}$=3288, 2936, 1641, 1525, 1505, 1464, 1435, 1398, 1364, 1308, 1252, 1204, 1165, 1099, 1061, 1005, 886, 813, 775, 750, 711, 667, 596, 467 cm^{-1}; HRMS: *m/z*: calcd for $C_{37}H_{55}N_{11}O_9+H^+$: 798.42570; found: 798.42645 $[M+H]^+$.

Im-Py-Py-GABA-PyPyPy-β-Ala-C$_3$-N(Me)-C$_3$-GABA-NH$_2$ (***16***): Compound **15** (0.25 g, 0.31 mmol) was added to a suspension of Pd/C (0.10 g, 10% on active charcoal) in anhydrous MeOH (20 mL). The mixture was stirred in a steel autoclave under H_2 (40 bar) for 2 h at 50 °C. The catalyst was removed by filtration through Celite, and the solvent was evaporated. After drying of the product in vacuo, the product was dissolved in anhydrous DMF. The resulting solution was added dropwise to a solution of **8b** (0.16 g, 0.35 mmol) in abs. DMF (5 mL), followed by DIEA (0.07 mL, 0.41 mmol) and HOBt (0.06 g, 0.39 mmol). The solution was heated to 60 °C, and DIC (0.06 mL, 0.40 mmol) was added. The solution was stirred for 3 h at 60 °C. Afterwards the solution was added to a sat. $NaHCO_3$/water mixture (1:4, 25 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, and the solvent was evaporated. The crude product was purified by column chromatography (EtOAc/MeOH 1:1+1% NEt_3). Dry MeOH (1 mL) was cooled to 0 °C, and acetyl chloride (50 μL) was slowly added dropwise, followed by stirring for 30 min. Boc-protected **16** (0.21 g, 0.17 mmol) was dissolved in anhydrous MeOH (1 mL), added dropwise to the acidic MeOH solution, and stirred at 0 °C for 30 min, followed by 60 min at ambient temperature. When the reaction was complete, the solvent was evaporated, and the residue was dried in vacuo. Compound **16** was obtained as a yellowish foam (0.15 g, 43%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=10.47 (s, 1H; N*H*), 9.91–9.84 (m, 3H; N*H*), 9.42 (br s, 1H; N*H*), 8.07–8.05 (m, 4H; N*H*), 7.79 (br s, 3H; NH_3), 7.42–6.88 (m, 12H; Py-*H*, Im-*H*), 4.00 (s, 3H; CH_3), 3.85–3.80 (m, 15H; CH_3), 3.39–3.37 (m, 2H; CH_2), 3.25–3.19 (m, 2H; CH_2), 3.11–3.00 (m, 8H; CH_2), 2.80–2.73 (m, 5H; CH_2, CH_3), 2.38–2.26 (m, 4H; CH_2), 2.18 (t, J= 7.2 Hz, 2H; CH_2), 1.77–1.73 ppm (m, 8H; CH_2); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ=171.53, 171.01, 169.26, 161.31, 161.28, 158.46, 158.01, 155.77, 138.66, 126.56, 126.32, 123.07, 122.95, 122.77, 122.71, 122.17, 122.13, 122.07, 122.05, 121.35, 118.64, 118.49, 118.45, 118.15, 117.94, 117.83, 104.90, 104.29, 53.07, 38.59, 36.10, 36.05, 35.93, 35.70, 35.62, 35.58, 35.14, 33.30, 31.92, 25.70, 24.03, 23.96, 23.06 ppm; IR: $\tilde{\nu}$=3283, 3088, 2927, 1634, 1575, 1532, 1464, 1435, 1403, 1259, 1176, 1128, 1062, 1005, 833, 797, 777, 720, 706, 668, 606, 518, 478, 465 cm^{-1}; MS (ESI$^+$): *m/z*: calcd for $C_{53}H_{72}N_{18}O_9+H^+$: 1105.9; found: 1106.6 $[M+H]^+$.

*Im-Py-Py-GABA-PyPyPy-β-Ala-C$_3$-N(Me)-C$_3$-GABA-NH-bis(*tert*-butoxycarbonylamino)alcohol* (***18***): Acetyl chloride (2 mL) was slowly mixed at 0 °C with dry MeOH (5 mL). After the mixture had been kept at 0 °C for 30 min, a solution of Boc-protected **16** (250 mg, 0.207 mmol) in dry MeOH (1 mL) was added, and the mixture was stirred for 30 min at 0 °C and for 30 min at room temperature. After removal of the solvent and drying in vacuo, the residue of **16** was dissolved in anhydrous DMF (1 mL) and added to a solution of **17** (130 mg, 0.241 mmol), DIEA (0.64 mL, 3.73 mmol), and HOBt (40 mg, 0.26 mmol) in anhydrous DMF (4 mL). The solution was heated to 60 °C, and DIC (80 μL, 0.52 mmol) was added. The solution was stirred for 3 h at 60 °C. Afterwards it was added to a mixture of sat. $NaHCO_3$/water (1:4, 50 mL) and CH_2Cl_2 (20 mL). The layers were separated, and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, and the solvent was evaporated. The crude product was purified by column chromatography (EtOAc/MeOH 1:1+1% NEt_3) to afford **18** as an off-white foam (210 mg, 62%). MS (ESI$^+$): *m/z*: calcd for $C_{82}H_{104}N_{20}O_{16}+H^+$: 1625.8; found: 1625.2 $[M+H]^+$.

Im-Py-Py-GABA-PyPyPy-β-Ala-C$_3$-N(Me)-C$_3$-GABA-NH-bis(guanidinium)-alcohol (***1***): Dry MeOH (2 mL) was cooled to 0 °C, and acetyl chloride (500 μL) was slowly added dropwise, followed by stirring for 30 min. Compound **18** (60 mg, 37 μmol) was dissolved in anhydrous MeOH (1 mL), added dropwise to the acidic MeOH solution, and stirred at 0 °C for 30 min, followed by 60 min at ambient temperature. When the reaction was complete, the solvent was evaporated, and the residue was dried in vacuo. The deprotected amine was dissolved in a MeOH/NEt_3 mixture (2:1, 6 mL). 4,5-Dihydroimidazole-2-sulfonic acid (22 mg, 0.149 mmol) was added, and the resulting solution was stirred for 16 h. After HPLC purification (Reprosil AQ C_{18} 5 μm, 250 mm, isocratic, 0.1% aqueous TFA+acetonitrile 74:26, 9 mL min^{-1}), **1** was obtained as a yellowish foam (11.2 mg, 19%). MS (ESI$^+$): *m/z*: calcd for $C_{78}H_{96}N_{24}O_{12}+H^+$: 1561.8; found: 1561.2 $[M+H]^+$.

DNA duplex stabilization by compound 1: All experiments were executed with the same 11-mer DNA duplex as previously reported for study of the interaction with hairpin polyamide **19**: d(CATTGTTAGAC)$^{3'}$:d(GTCTAACAATG)$^{3'}$.[18] UV: JASCO V-650, conditions: sodium cacodylate (pH 7.0, 10 mM), KCl (10 mM), $MgCl_2$ (10 mM), $CaCl_2$ (5 mM); profiles were measured at 260 nm, heating rate 1 °C min^{-1}, 3 cycles, duplex (5 μM) and **1** (5 μM). CD titrations: JASCO AKS J-810, conditions: sodium cacodylate (pH 7.0, 10 mM), KCl (10 mM), $MgCl_2$ (10 mM), $CaCl_2$ (5 mM), 20 °C, duplex (5 μM), and incremental addition of **1**.

DNA cleavage assay: Plasmid preparation, sample incubation, and electrophoretic analysis followed the previously published methods exactly.[14b]

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