Typical CI source conditions were as follows: source temperature 100 °C; repellor voltage 0.0 V; ion-extraction voltage 8 kV; source pressure 0.1–0.3 Torr. The NR experiments were performed in the first of the collision cells positioned between the magnet and the second electrostatic analyzer, using Xe or CH_4 as the neutralizing colliders, at a pressure adjusted to achieve a 80 % transmittance. Reionization was achieved utilizing O_2 as the collider, approximately at the same transmittance. Any ions remaining after the first collision event were deflected from the primary neutral beam using a high-voltage electrode (1 kV), whose efficiency was checked by suitable control experiments. The NR spectra were averaged over 20–50 acquisitions to achieve a satisfactory signal-to-noise ratio.

The gases were of research grade from commercial sources with a stated purity exceeding 99.95 mol % and were used without further purification. Ozone was prepared from dry O_2 in a commercial ozonizer, collected in a silica trap at 77 K and recovered by controlled warming of the trap. $^{18}O_2$ and $H_2{}^{18}O$ (> 99 ^{18}O atom %) were obtained from Isotec (Miamisburg, USA). The $(H_2O \cdot O_2)^+$ ion was prepared by hydration of O_2^+ in the positive O_2/CI of water[11]. Much higher yields were achieved by displacement of O_3 by H_2O in the O_5^+ complex[12], a very efficient process as demonstrated by kinetic experiments performed utilizing a Fourier-transform ion-cyclotron resonance mass spectrometer (47e Apex, Bruker Spectrospin AG, Bremen, Germany). The $(H_2O \cdot O_2)^-$ ion was prepared by hydration of O_2^- in the negative CI of moist oxygen.[13]

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Efficient Hydrolysis of RNA by a PNA–Diethylenetriamine Adduct**

Jeroen C. Verheijen, Birgit A. L. M. Deiman, Esther Yeheskiely, Gijs A. van der Marel, and Jacques H. van Boom*

A few years ago, Komiyama et al. reported[1] that a hybrid composed of diethylenetriamine (DETA) anchored to the 5′-end of DNA by means of a urethane bond (Figure 1a) hydrolyzed linear RNA selectively at the 3′-side of cytosine 22 (C22, marked with an arrow) to give a 22-mer RNA fragment

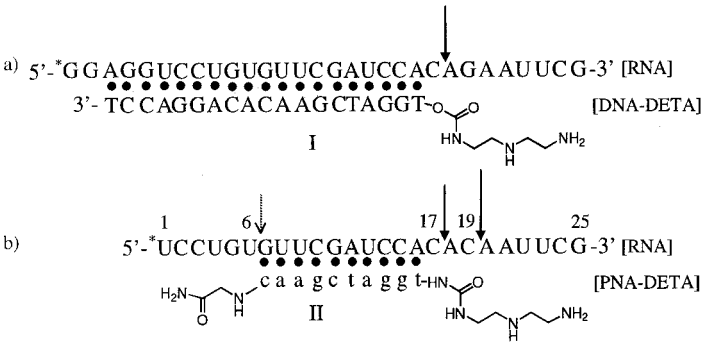

Figure 1. a) Complex **I** of RNA and DNA–DETA as employed by Komiyama et al.; b) complex **II** of RNA and PNA–DETA. Arrows indicate cleavage positions of RNA for hydrolysis by DNA–DETA or PNA–DETA. Nucleotide units are written in uppercase, PNA units in lowercase.

with a 3′-phosphate terminus. This selective scission was ascribed to intramolecular acid–base cooperation of an ammonium ion and a neutral amine in the ethylenediamine moiety $[N(CH_2)_2NH_2]$ of the DNA–DETA adduct.[2] Interestingly, the total conversion for the RNA hydrolysis was only 10 mol % after incubation of the RNA·DNA–DETA complex **I** (Figure 1a) at pH 8 for 4 h at 50 °C. The relatively low conversion of the RNA substrate may be attributed to less effective hydrogen bonding in complex **I**. We surmised that a higher conversion could be attained by decreasing significantly the freedom in dangling motion of the duplex in the complex.

It is generally accepted that peptide nucleic acids (PNAs)[3] nicely mimic the physical properties of DNA. Thus, PNAs hybridize sequence specifically to RNA (DNA). They bind, however, more strongly to RNA (DNA)[4] due to the presence of a neutral polyamide backbone. The latter features, together

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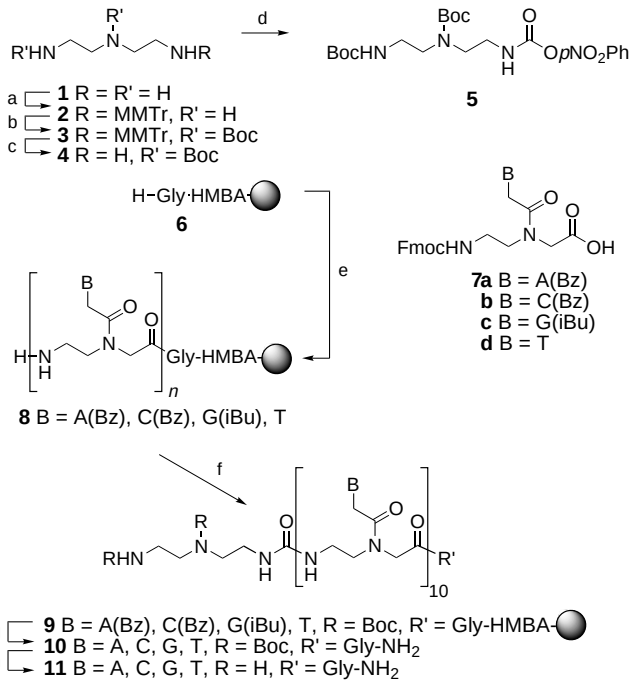
with the fact that PNAs resist biodegradation,[5] were an incentive to replace the DNA – DETA adduct in complex **I** by a PNA – DETA adduct as in complex **II** (Figure 1b).

As part of an ongoing program[6] towards the design and use of antisense probes based on PNA, we here report the preparation and ribonucleolytic properties of a PNA attached by means of a urea bond to DETA.

Based on the RNA substrate employed by Komiyama et al. (Figure 1a), a 25-mer RNA (Figure 1b) containing a 10-mer sequence (i. e. G7 – A16) complementary to the adduct, as well as two potential neighboring C – A scission sites[7] adjacent to A16, was chosen as the target RNA. The RNA 25-mer, prepared according to a well-established solid-phase approach,[8] was ^{32}P-labeled at the 5′-end using adenosine 5′-[γ-^{32}P]-triphosphate and T4 polynucleotide kinase. The PNA – RNA complex in which the diethylenetriamine moiety in PNA **11** is replaced by an acetyl group (PNA-Ac) showed a T_m of 69 °C (pH 7, 100 mM NaCl), indicating that the PNA – DETA adduct forms a sufficiently stable duplex with the target RNA.

The construction of the 10-mer PNA – DETA adduct **11** commences with the four-step transformation of DETA (**1**) into the *N*,*N*′-Boc-protected and activated derivative **5** (Scheme 1). Treatment of excess **1** in dichloromethane with MMTrCl (see Scheme 1 for abbreviations) led to the isolation of the crude mono-MMTr derivative **2**. Treatment of the latter with excess Boc_2O in dioxane gave, after purification on silica gel, the fully protected derivative **3**. Removal of the *N*-MMTr group of **3** with TFA in the presence of the scavenger



Scheme 1. a) Monomethoxytrityl chloride (MMTrCl), CH_2Cl_2; b) Boc_2O (di-*tert*-butyldicarbonate), dioxane, 73% (2 steps); c) 5% trifluoroacetic acid (TFA), Bu_3SnH, CH_2Cl_2, 91%; d) $pNO_2PhOC(O)Cl$, Et_3N, CH_2Cl_2, 63%; e) stepwise elongation with **7a** – **d**; f) **5**, *N*,*N*-diisopropylethylamine (DIPEA), DMF; g) NH_3/MeOH, 50 °C; h) 75% TFA, H_2O. Boc = *tert*-butyloxycarbonyl, Fmoc = fluorene-9-ylmethoxycarbonyl, HMBA = *para*-hydroxymethylbenzoic acid. The polystyrene support is represented by shaded circles.

nBu$_3$SnH resulted, after extraction, in the isolation of the crude free amine **4**. Reaction of **4** with *para*-nitrophenyl chloroformate led, after purification on silica gel, to the activated derivative **5** in an overall yield of 42%.

At this stage, the PNA decamer complementary to the G7 – A16 stretch of the RNA in complex **II** was assembled following a well-established solid-phase synthesis.[6] Thus, sequential elongation of glycine anchored by an ester bond to functionalized polystyrene beads (i. e. **6** in Scheme 1) with Fmoc/*N*-acyl-protected building blocks **7a** – **d**[9] under the agency of the coupling reagent HATU and subsequent reaction of the resulting resin-bound 10-mer PNA **8** with excess **5** in the presence of DIPEA for 16 h at 20 °C gave immobilized **9** (see Experimental Section). Removal of the *N*-acyl protecting groups from the nucleobases in **9** and concomitant release from the solid support was effected by ammonolysis (NH_3/MeOH) to give, after purification by reversed-phase high-performance liquid chromatography (RP-HPLC), the homogeneous *N*,*N*′-Boc protected PNA – DETA adduct **10**. Treatment of the latter adduct with TFA (75%) resulted in the isolation of the fully deprotected PNA – DETA adduct **11**, the homogeneity and identity of which was firmly established by RP-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Having the 10-mer PNA – DETA adduct **11** and the 25-mer RNA in hand, we examined the ribonucleolytic behavior of **11** by degrading the target 25-mer RNA at 40 °C in a Tris · HCl (10 mM) buffer containing NaCl (100 mM) and EDTA (0.1 mM) at pH 7. In this respect, it is of interest to note that the pH is one unit below that required for optimal cleavage of the phosphodiester bonds in RNA by intramolecular acid – base cooperation. The hydrolysates were analyzed by electrophoresis on 20% denaturing polyacrylamide gel.

A typical electrophoresis pattern is presented in Figure 2. Cursory inspection of the traces in lanes 4 – 8 shows, after a

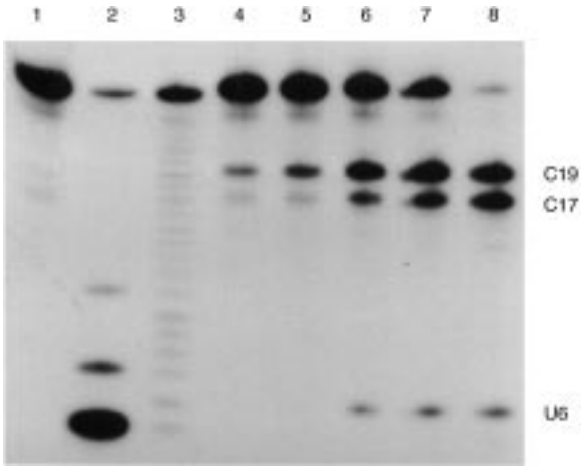


Figure 2. Autoradiograph for the cleavage of the 5′-labeled RNA 25-mer in Tris · HCl buffer at pH 7 and 40 °C in the presence of EDTA (0.1 mM). Lane 1: PNA – DETA (t = 0); lane 2: T1 digestion; lane 3: alkaline hydrolysis ladder; lane 4: PNA – DETA (t = 1 h); lane 5: PNA – DETA (t = 2 h); lane 6: PNA – DETA (t = 4 h); lane 7: PNA – DETA (t = 8 h); lane 8: PNA – DETA (t = 24 h). $[RNA]_0$ = 60 nM, [PNA – DETA] = 2 μM. EDTA = ethylenediaminetetraacetic acid, Tris = tris(hydroxymethyl)aminomethane.

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lapse of 24 h, the presence of two major 5′-end labeled RNA fragments. Comparison of the mobility of the two cleavage products with T1 digestion (lane 2) and alkaline hydrolysis (lane 3) of the target RNA (lane 1) revealed that both fragments resulted from hydrolysis at the 3′-side of C17 and C19, respectively. The total conversion for the RNA hydrolysis was approximately 29 mol% after 4 h at 40 °C, as gauged by densitometry (Figure 3). It is also worthwhile mentioning that the cleavage of the phosphodiester bond in close proximity of the duplex structure in complex **II** is relatively less efficient (see, for instance, Figure 3). A similar preference for the more distant cleavage site was also reported in other, closely related studies.[7a,b, 10]

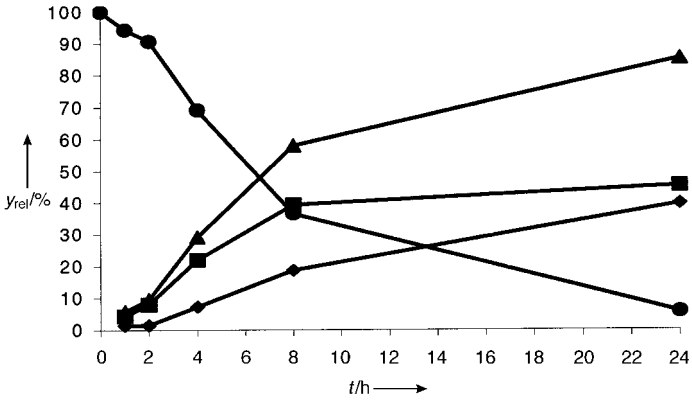

Figure 3. Relative amounts (y) of intact and cleaved RNA at different reaction times. Intact RNA 25-mer (●), fragment 1 (RNA 19-mer) (■), fragment 2 (RNA 17-mer) (◆), summed amount of fragments 1 and 2 (▲).

In contrast with the latter observations, no scission was observed when the 10-mer PNA-Ac was used instead of the PNA–DETA conjugate **11**. The same holds for incubation of the 25-mer RNA with diethylenetriamine in the presence or absence of the PNA-Ac decamer, thus illustrating the sequence specificity of RNA hydrolysis by the PNA–DETA adduct **11**.

Apart from the two main cleavage products an additional minor degradation product, resulting from scission after U6, could be observed upon prolonged incubation (see lanes 6–8). Since participation of the glycinamide in the cleavage is ruled out by the inactivity of the PNA-Ac, the formation of the latter fragment may indicate that the spatial arrangement of the RNA·PNA–DETA complex **II** positions the ethylenediamine residue of the PNA–DETA in close proximity (i. e. approximately one helical turn) to the phosphodiester bond between U6 and G7 in the RNA substrate.

These results show for the first time that a PNA-based adduct cleaves an RNA sequence specifically at micromolar concentration under physiological conditions. An additional but nonetheless essential feature of this type of adduct is the fact that almost complete conversion of RNA can be attained (see e. g. Figure 3) with a relatively short stretch of PNA, thus obviating the need for an excessively long sequence of DNA to reach the same goal.[11] In this respect, we note that our PNA approach nicely complements the recently reported selective cleavage of HIV-1 TAR-RNA with a peptide–cyclen conjugate.[12] In summary, the ease of preparing artificial nucleases based on PNA will be of great interest for the future design of other types of chemical nucleases.

Experimental Section

Solid-phase synthesis of PNA–DETA adduct **11**: All solvents (Biosolve, DNA synthesis grade) were used as received. Solid-phase synthesis was performed on a Pharmacia Gene Assembler using highly cross-linked polystyrene beads as the solid support (loading: 26–28 μmol g^{-1}) on a 1 μmol scale. The support was functionalized with a glycine moiety attached to a *para*-hydroxymethylbenzoic acid linker. Assembly of the PNA part was established using solutions of 0.3 M of monomers **7a–d**, 0.3 M DIPEA and 0.3 M HATU (*O*-(azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in acetonitrile/dimethylformamide (1/1 *v*/*v*). Prior to coupling, the monomers were preactivated for 1 min by mixing equal amounts of the PNA monomer (15 equiv per μmol support), HATU and DIPEA solutions. The protocol for one PNA chain extension cycle consisted of 1) wash: acetonitrile/dimethylformamide (1/1 *v*/*v*), 2.5 mL; 2) coupling: PNA + HATU + DIUPEA in acetonitrile/dimethylformamide (1/1 *v*/*v*), 15 min; 3) wash: acetonitrile/dimethylformamide (1/1 *v*/*v*), 2.5 mL; 4) capping: Ac_2O/lutidine/*N*-methylimidazole/tetrahydrofuran (1/1/1/7 *v*/*v*/*v*/*v*), 2.0 mL; 5) wash: acetonitrile/dimethylformamide (1/1 *v*/*v*), 2.5 mL; 6) Fmoc removal: 20% piperidine in acetonitrile/dimethylformamide (1/1 *v*/*v*), 3 min; 7) wash: acetonitrile/dimethylformamide (1/1 *v*/*v*), 2.5 mL. After the last elongation step, the solid support was transferred to a separate reaction vessel, and a solution of **5** (12 mg, 25 μmol) and diisopropylethylamine (5 μL) in dimethylformamide (0.5 mL) was added. After mixing for 16 h, the reagents were drained from the vessel, and the solid support was washed with dimethylformamide (2 mL) followed by dichloromethane (2 mL). At this stage, the oligomers were cleaved from the support with concomitant deprotection of the exocyclic amino groups by treatment with methanolic ammonia (1.5 mL) at 50 °C for 16 h. RP-HPLC purification and analysis were carried out on a Jasco HPLC system equipped with a LiChrospher 100 RP-18 end-capped column (10.0 × 250 mm and 4.0 × 250 mm, respectively). Gradient elution was performed at 40 °C by building up a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile/water, 9/1 *v*/*v*) with a flow rate of 1.0 mL min^{-1}. The Boc groups of purified **10** were removed by treatment with 75% TFA. Homogeneous deprotected PNA–DETA adduct **11** was obtained after freeze-drying. The identity and homogeneity of oligomers **10** and **11** were confirmed by MALDI-TOF MS and RP-HPLC. **10**: MALDI-TOF MS: *m*/*z*: 3139.1 $[M+H]^+$, 3160.2 $[M+Na]^+$; calcd for $C_{125}H_{165}N_{65}O_{35}$: *m*/*z*: 3138.1. **11**: MALDI-TOF MS: *m*/*z*: 2939.6 $[M+H]^+$, 2962.3 $[M+Na]^+$; calcd for $C_{115}H_{149}N_{65}O_{31}$: *m*/*z*: 2937.9.

RNA degradation with PNA–DETA adduct **11**: All buffers were made from highly purified Milli Q water and were sterilized before use. Labeled RNA 25-mer and PNA–DETA were mixed in Tris·HCl buffer (10 mM, pH 7) containing NaCl (100 mM) and EDTA (0.1 mM) to give the following concentrations: [RNA] = 60 nM, [PNA–DETA] = 2 μM. The samples (70 μL) were incubated at 40 °C. After incubation, the RNA was immediately precipitated by addition of 3 M NaOAc (pH 5, 7 μL), EtOH (225 μL), and 10 μg μL^{-1} tRNA (1 μL). The precipitated RNA was recovered by centrifugation, and was then dried and redissolved in 5 μL water and 5 μL loading buffer. The solutions were heated at 80 °C for 1 min, centrifuged and analyzed on a 20% denaturing electrophoresis gel.

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Synthesis of Polycyclic Ethers by Two-Directional Double Ring-Closing Metathesis**

J. Stephen Clark* and Olivier Hamelin

Neurotoxic marine polycyclic ethers of the brevetoxin and ciguatoxin family continue to be interesting synthetic targets as a consequence of their architectural complexity and potent biological activity.[1] They constitute a major challenge in terms of medium-size ring construction as illustrated by CTX-3C (Figure 1).[2] This ciguatoxin contains a total of 13 rings and possesses a daunting combination of saturated and unsaturated medium-sized rings. The size of the brevetoxins and ciguatoxins, coupled with their structural and stereochemical complexity, has made them irresistible synthetic

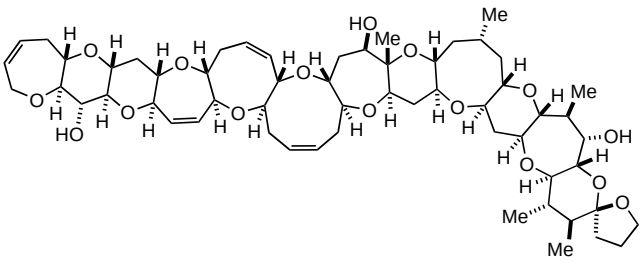
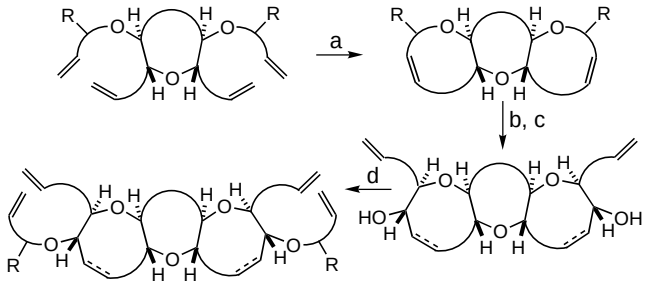


Figure 1. The structure of CTX-3C.

targets, providing a stimulus for the development of many new reactions and strategies for the construction of polycyclic systems.[3] This work has culminated in the recent total syntheses of brevetoxins A and B, completed by Nicolaou and co-workers,[4] as well as several syntheses of the smaller congener hemibrevetoxin B.[5]

We recently presented a powerful strategy for the construction of subunits found in the brevetoxins and ciguatoxins. Catalytic ring-closing metathesis (RCM)[6] reactions of enol ethers,[7a,b] allylic ethers,[7c,d] or alkynyl ethers[7e] were promoted by the Schrock catalyst [2,6-*i*Pr$_2$C$_6$H$_3$NMo{OC(CF$_3$)$_2$Me}$_2$CHCMe$_2$Ph] (**1**)[8] or the Grubbs catalyst [Cl$_2$\{(*c*-C$_6$H$_{11}$)$_3$P\}$_2$RuCHPh] (**2**),[9] leading to the formation of cyclic ethers.[10] Stereoselective elaboration of the RCM products then gave fully functionalized, saturated or unsaturated, cyclic ethers suitable for conversion into polyoxacyclic units of the type found in the natural products.[7d]

We now report a new strategy for polycyclic ether assembly, the essence of which is illustrated in Scheme 1.[11] We envisaged the construction of rings in a two-directional



Scheme 1. Strategy for the synthesis of polycyclic ethers using two-directional RCM reactions. a) Ring-closing metathesis; b) ring functionalization; c) side-chain functionalization; d) side-chain introduction.

manner by performing double RCM reactions of allylic ethers, enol ethers, or alkynyl ethers, or any combination thereof.[12] We expected that the reaction would be of general applicability, permitting the preparation of tricyclic ethers with any permutation of ring sizes. Elaboration of the tricyclic products, using our recently described methods,[7b] followed by side-chain functionalization would then provide appropriately functionalized systems for iteration of the two-directional ring-closure sequence.

The precursors were prepared as shown in Scheme 2. Treatment of commercially available tri-*O*-acetyl-D-glucal (**3**) with sodium methoxide afforded D-glucal, which was converted into 4,6-*O*-di-(*tert*-butyl)silanediyl-D-glucal accord-

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