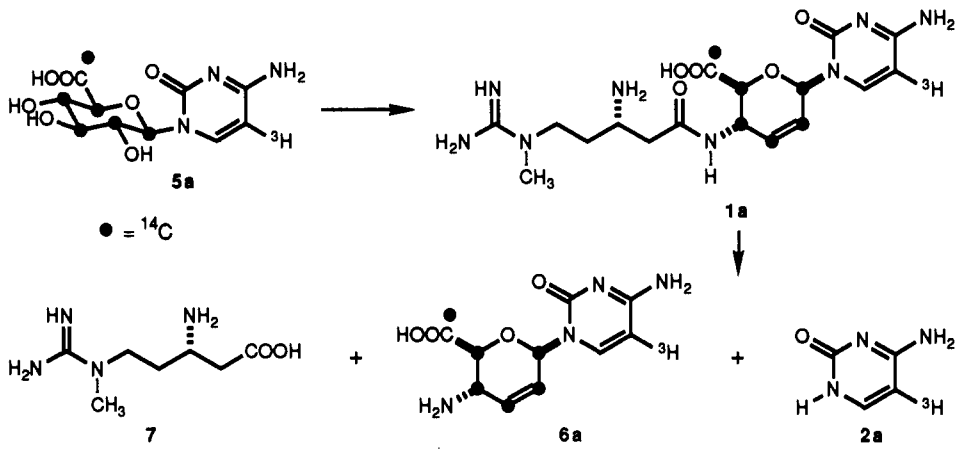
Scheme II



spectrum. As is common for nucleosides, **5** was not readily taken up by *S. griseochromogenes* mycelia (HPLC analysis), and efforts to prepare a cell-free extract or protoplasts that could metabolize **5** to **1** were unsuccessful. In order to test **5** as a biosynthetic intermediate, a sample, **5a** ($^3H/^{14}C$ = 4.25, 1.29 × 10^8 dpm/mmol ^{14}C), was prepared enzymatically from [5-^{3}H]cytosine (52.45 μCi, 19.9 mCi/μmol, Sigma Chemical Co.) and UDP[U-^{14}C]-D-glucuronic acid (10.95 μCi, 250 μCi/μmol, ICN). A portion of this (10.0 mg, 4.49 × 10^6 dpm ^{14}C) was fed to a 200-mL fermentation in the standard complex medium[14] 39 h after inoculation with a seed culture. Ninety-eight hours later workup[14] yielded 455 mg of labeled blasticidin S **1a** ($^3H/^{14}C$ = 6.75, 1.01 × 10^4 dpm/mmol ^{14}C). Most of **5a** was recovered from the broth unchanged (88.4%). On the basis of the unrecovered material, the ^{14}C incorporation was 1.8%. The change in $^3H/^{14}C$ could be explained if only 0.14% of the **5a** fed had been hydrolyzed to [5-^{3}H]cytosine, **2a**, since **2** had previously been incorporated almost quantitatively.[8] To test the specificity of the incorporation, 300 mg of **1a** were hydrolyzed[2,3] yielding labeled cytosinine **6a** (29.4 mg, $^3H/^{14}C$ = 6.26, 9.70 × 10^3 dpm/mmol ^{14}C, 96% retention of ^{14}C and 89% retention of ^3H from **1a**), labeled cytosine **2a** (21.4 mg, 5.84 × 10^4 dpm/mmol ^3H, 86% retention of tritium), and blasticidic acid, **7** (not isolated, but unlabeled vide supra). Some exchange of tritium had occurred during the hydrolysis. These results demonstrate unequivocally the specific intact incorporation of **5a** exclusively into the cytosinine portion of blasticidin S, **1**.

$$\mathbf{5a} \rightarrow \mathbf{1a} \rightarrow \mathbf{6a} + \mathbf{2a} + \mathbf{7}$$

The biosyntheses of a number of nucleoside antibiotics have been studied.[24-27] The biosynthesis of blasticidin S appears to be the first instance where the formation of a novel nucleoside has been demonstrated at the cell-free level to be the first committed step in the secondary pathway. In addition, although UDPglucuronosyl transferases are common in mammalian xenobiotic metabolism and at least two have been reported in fungi (also eukaryotes),[28,29] this is the first report of such an enzyme from a prokaryotic organism.[30]

Acknowledgment. Dr. Y. Miyazaki, Kaken Chemical Co., Ltd., Japan, is thanked for providing a culture of *S. griseochromogenes* and recipes for the standard seed and production media. This work was supported by Public Health Service Research Grant GM 32110 to S.J.G. The Bruker AC 300 NMR spectrometer used in this work was purchased in part through grants from the Public Health Service Division of Research Resources (RR04039-01) and the National Science Foundation (CHE-8712343) to Oregon State University.

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Oligoamines as Simple and Efficient Catalysts for RNA Hydrolysis

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Recent interest has focused on the molecular design of artificial nucleases, in which catalytic residues are attached to sequence-recognizing moieties.[1] Developments have been made in oxidative fission of specific ribose residues in DNA.[2] However, cleavage of DNA and RNA via hydrolysis of the phosphodiester linkage has been scarcely accomplished.[3-6] We report here remarkable

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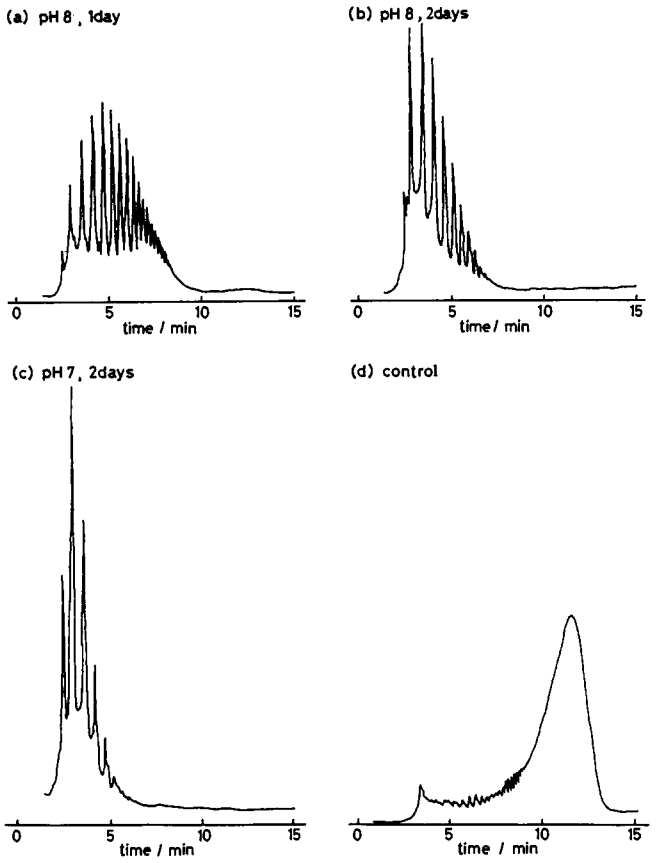

Figure 1. HPLC patterns for the hydrolysis of poly(A) at 50 °C by ethylenediamine (N-N; 1.0 M): (a) pH 8, 1 day; (b) pH 8, 2 days; (c) pH 7, 2 days; (d) control (without the amine, 2 days at pH 8); other reaction conditions and separation conditions are shown in ref 8.

acceleration of RNA hydrolysis by simple oligoamines as highly potent catalytic moieties for artificial ribonucleases.

Figure 1 depicts typical ion-exchange HPLC patterns for the hydrolysis of poly(A) (degree of polymerization (DP): 1100) at 50 °C.[7,8] With ethylenediamine (N-N; 1.0 M) at pH 8, the peaks gradually shifted toward shorter retention time (compare patterns a and b with pattern d as control), showing prompt degradation of poly(A). After 2 days, only small oligomers (mostly 5-mer or smaller) were perceived (b). At $[N\text{-}N]_0$ = 3.0 M, monomer and dimer alone were present after 2 days. The diamino character of N-N is definitely required for effective catalysis, since ethylamine (N_1; 2.0 M) caused only a small change in HPLC. Without the amine catalyst, virtually no hydrolysis took place (d). All the hydrolysis proceeded homogeneously.

When hydrolysis by N-N was achieved at pH 7, the reaction mixture was slightly turbid in the early stage, due to formation of a polyion complex (pK_a's of N-N are 6.5 and 9.2). Two days later, however, the turbidity was completely eliminated, reflecting degradation of poly(A) to small fragments. It is noteworthy that hydrolysis is faster at pH 7 than at pH 8 (compare b with c). Gradual disappearance of turbidity by rapid degradation of poly(A) was also observed for hydrolysis at pH 8 with triethylenetetramine (N_4) or pentaethylenehexamine (N_6).

Table I. Hydrolysis of RNAs by oligoamines[a]

RNA	catalyst	% of phosphodiester bonds hydrolyzed[b]
poly(A)	N-N	33 (26)[c]
		48[d]
		69[e]
	N_4	20 (12)[c]
	N_6	31
	N_1	3 (0)[c]
poly(C)	N-N	16
poly(U)	N-N	28
poly(G)	N-N	0

[a] At pH 8, 50 °C for 2 days; $[N_1]_0$ = 2.0 M, $[N\text{-}N]_0$ = 1.0 M, $[N_4]_0$ = 0.5 M, and $[N_6]_0$ = 0.33 M unless otherwise noted. In the absence of the amine catalyst, hydrolysis of RNAs did not proceed to a measurable extent. [b] Error limits are around 2%. [c] The values in parentheses refer to the hydrolysis of oligo(A) (40-mer obtained through a DNA synthesizer). [d] At pH 7 ($[N\text{-}N]_0$ = 1.0 M). [e] $[N\text{-}N]_0$ = 3.0 M at pH 8.

Catalytic activities of the amines in the hydrolysis of poly(A), as well as in the hydrolysis of oligo(A) (40-mer prepared by DNA synthesizer),[9] have been quantitatively evaluated (Table I).[10] The activities of the oligoamines N-N, N_4, and N_6 largely surpass the value for N_1. Notably, catalytic hydrolysis of oligo(A) is successful only by the oligoamines, since N_1 is inactive. That intramolecular cooperation between two amino residues plays a dominant role is strongly evidenced.

The hydrolysis rate at pH 8 monotonously increased with $[N\text{-}N]_0$, and about two-thirds of the phosphodiester bonds were hydrolyzed for 2 days at 3.0 M. The pH–rate constant profile indicated that monocationic species $H_3N^+(CH_2)_2NH_2$ is responsible for the catalysis.[11] Below pH 7 the catalysis is additionally promoted by formation of the polyion complex, in which the amine functions efficiently as an "intracomplex" catalyst.[12]

Catalysis of N-N is also effective for the hydrolysis of poly(C) and poly(U) (see Table I). However, poly(G) is hardly hydrolyzed by N-N. Apparently, the efficiency of the catalysis is dependent on the higher order structure of RNA. Under the present conditions, poly(A) and poly(C) take single-stranded helices and poly(U) takes random form, whereas the favorable conformation of poly(G) is a four-stranded helix.[13,14]

The present hydrolysis proceeds via the rate-determining formation of 2′,3′-cyclic phosphates of ribonucleotides as intermediates, as confirmed by the lack of catalysis of the oligoamines for the hydrolysis of poly(dA). General base catalysis by neutral amine and general acid catalysis by protonated amine intramolecularly cooperate, facilitating nucleophilic attack of the 2′-OH toward the phosphorus atom.[15-17] The mechanism is in accord

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(7) The DP value of poly(A), purchased from Sigma Chemical Co., was estimated to be 1100 ± 200 by electrophoresis using φX174 *Hae*III and λ *Hin*dIII restriction enzyme fragments as the internal standard.

(8) Hydrolysis of RNA was carried out at pH 8 (Tris 0.05 M buffer), 50 °C with RNA residual concentration of 0.005 M unless otherwise noted. Special caution was exercised to avoid contamination of ribonuclease. Ion-exchange HPLC was carried out on a DEAE-NPR column of TOSOH Co. with a linear gradient from 0 to 0.4 M $NaClO_4$ at pH 9 in 0.02 M Tris. Participation of any divalent metal ion in the present catalysis was ruled out by the fact that EDTA showed no effect on the hydrolysis rate.

(9) Hydrolysis of the 40-mer, containing smaller oligomers as minor by-products, at pH 8 by N-N and N_4 as well as by N_1 proceeded homogeneously.

(10) The number of phosphodiester bonds in the reaction mixture at a given reaction time was evaluated by HPLC using a calibration curve of DP vs retention time. The curve was obtained from poly(A)'s of DP 2, 12, and 40 (synthesized by use of a DNA synthesizer) as well as those of DP 520 and 1100. Poly(A) of DP 520, prepared by sonication of commercially obtained poly(A), was a cordial gift from Professors Mitsuru Akashi and Eiji Yashima at Kagoshima University, which we acknowledge gratefully. Hydrolysis of other RNAs was analyzed in a similar way.

(11) The profile was satisfactorily interpreted in terms of the catalysis both by the monocationic species and by the neutral species (see refs 15 and 16), although positive deviation due to polyion complex formation was observed below pH 7. The contribution of the neutral species to the catalysis at pH 7–8 is marginal.

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with the cooperation of two imidazoles in ribonuclease, precisely studied by Breslow using model systems.[4] Contamination of ribonuclease is ruled out by formation of ribonucleoside 3′-phosphate and the 2′-phosphate as the final products in almost a 1:1 ratio.[18] Ribonuclease should produce, if any, the 3′-phosphate in 100% selectivity.[19]

In conclusion, oligoamines N-N, N_4, and N_6 have large catalytic activities for RNA hydrolysis, together with simplicity in structure and stability, being promising as catalytic sites for artificial ribonucleases. Attachment of the oligoamines to sequence-recognizing moieties is now in progress.

Acknowledgment. We thank Dr. Kazunari Taira at Fermentation Research Institute, Agency of Industrial Science and Technology, for valuable comments. This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

(17) Furthermore, positive charges of N_4 and N_6 might electrostatically stabilize the transition state of hydrolysis, similarly to the function of lysine 41 of ribonuclease A (Deakyne, C. A.; Allen, L. C. *J. Am. Chem. Soc.* **1979**, *101*, 3951). Participation of the third amino residue (either cationic or neutral) in the catalysis is also plausible.

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Cooperative Binding of Oligonucleotides to DNA by Triple Helix Formation: Dimerization via Watson–Crick Hydrogen Bonds

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Cooperative binding by proteins to DNA results in higher sequence specificity as well as greater sensitivity to concentration changes.[1] We recently reported cooperative binding of two oligonucleotides at abutting sites by triple helix formation on double helical DNA.[2] However, the enhanced binding observed was modest (a factor of 3.5) and likely due to favorable base-stacking interactions between adjacent oligonucleotides and/or induced conformational changes propagated to adjacent binding sites.[2] Thus, the issue arises whether cooperativity in oligonucleotide-directed triple helix formation can be enhanced by the addition of *discrete dimerization domains*. We report here the binding properties of oligonucleotides that dimerize by Watson–Crick hydrogen bonds and bind neighboring sites on double helical DNA by triple helix formation.

Our design for effecting cooperativity is a Y-shaped complex formed on double helical DNA by two oligonucleotides, each containing two separate functional domains, binding and dimerization.[3] Each oligonucleotide possesses a pyrimidine segment designed to recognize a specific purine duplex target site through the formation of Hoogsteen hydrogen bonds (TAT and CGC triplets).[5-7] These pyrimidine regions are 9 and 15 nucleotides in length and are designated as recognition domains A and D, respectively (Figure 1).[8] Additionally, each oligonucleotide possesses a segment of mixed sequence composition. These sequences, designated dimerization domains B and C (Figure 1), are complementary to allow formation of eight base pairs of duplex DNA held together by Watson–Crick hydrogen bonds. The recognition and dimerization domains of each oligonucleotide are connected by a linker base introduced for the purpose of providing conformational flexibility at the junction between triplex and duplex DNA.[9] On the basis of model building studies, the 9 and 15 base pair binding sites were separated by two base pairs in order to accommodate a Y-shaped motif (Figure 2). A series of oligonucleotides, **1–8**, was synthesized to evaluate the role of each domain (Figure 1). The modified base **T*** was incorporated at the 5′ termini of oligonucleotides **1–3**, each targeted to the nine base pair half site, to allow analysis by the affinity cleaving method.[5,10] Because T* is positioned at the 5′ terminus of domain A, these cleavage experiments monitor the binding of only those oligonucleotides directed to the nine base pair target site. With this experimental design, cooperative binding due to the presence

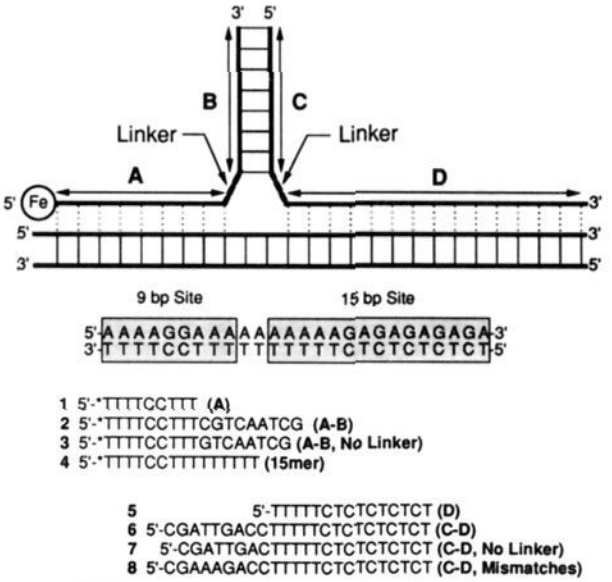

Figure 1. Schematic representation of a Y-shaped complex composed of two triplex forming oligonucleotides which cooperate through formation of a small segment of Watson–Crick double helical DNA. Two oligonucleotides possessing duplex recognition domains (A and D) and dimerization domains (B and C) connected by a linker base are designed to bind cooperatively to a double helical DNA target site. Thick solid lines represent the DNA backbone of the target site and associated oligonucleotides. Thin solid lines represent Watson–Crick hydrogen bonds, while dashed lines indicate Hoogsteen hydrogen bonds. The domains contained within each oligonucleotide are given in parentheses.

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(8) Binding sites 9 and 15 base pairs in size were chosen because at μM oligonucleotide concentrations (25 °C, pH 6.6) the former would be largely unoccupied while the latter nearly saturated. Since our experiments monitor the binding of oligonucleotides targeted to the 9 bp site, this arrangement maximizes any possible binding enhancement resulting from oligonucleotides present at the 15 bp site.

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