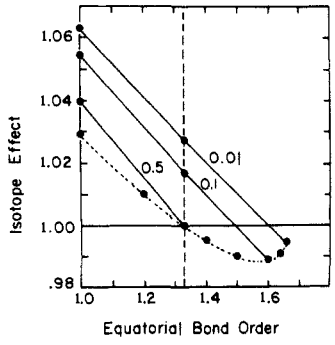

Figure 1. Calculated kinetic ^{18}O isotope effects at 100 °C for phosphate transfer. The reactant is a phosphate monoester with three ^{18}O in the nonbridge oxygens. The transition state is a trigonal bipyramid with the equatorial bond order shown. The vertical dashed line is the equatorial bond order in the reactant before reaction, and the dotted line connects points where the total bond order to P is 5 in the transition state. Solid lines connect points with the same axial bond order (numbers shown on figure).

^{18}O isotope effect for deprotonation of a phosphate monoester was determined in the previous paper[11] to be 1.0042 per ^{18}O at 100 °C, and thus correction for the preequilibrium proton transfer to the bridge oxygen in mechanisms 1 or 3 gives 1.0004 as the isotope effect on P–O bond cleavage (1.001 for three ^{18}O).

We show in Figure 1 isotope effects for phosphate ester hydrolysis calculated at 100 °C for ^{18}O-substitution in the three nonbridge oxygens.[12] If the mechanism is dissociative as believed, the observed isotope effect near unity suggests that total bond order to P is *not* conserved in the transition state. For an axial bond order of 0.1, the equatorial bond order would be 1.48, which corresponds to a total bond order to P of 4.64, or a net positive charge on P of 0.36. For an axial bond order of 0.01, the equatorial bond order would be 1.59, and the charge on P +0.21. The alternate explanation that the transition state is early is not possible in this case because the equilibrium constant for the reaction is near unity if the concentration of water is expressed in the same units as for the other reactants. These data do not address the question of whether metaphosphate is a free intermediate or whether the reaction is an S_N2 one with very low axial bond order in the transition state. Because K_{eq} is near unity and metaphosphate is very unstable relative to reactant or product, the transition states would be very similar for both mechanisms.

Acknowledgment. This work was supported by NIH Grant GM 18938 to W.W.C. W.B.K. holds a NIH postdoctoral fellowship (GM09677). NSF provided a major portion of the funding to purchase the isotope ratio mass spectrometer (PCM 8218027). We thank Isabel Treichel for technical assistance with isotope ratio mass spectrometry.

(9) The equation used to calculate ^{18}k (the ^{18}O isotope effect for single ^{18}O-substitution) was [10]

$$^{18}k = 1 + ([w/(^{13}k - [(1 - b)z/(bx)][w - {}^{13}k])]^{1/3} - 1)[1 + (1 - y)/3]$$

where w = observed isotope effect = 1.013, x = fraction of ^{13}C in the [1-^{13}C]glucose = 0.99, y = fraction of $^{18}O_3$ in the ^{13}C,^{18}O-containing glucose 6-phosphate = 0.85, z = fraction of ^{13}C in [1-^{12}C]glucose = 0.0001, b = fraction of ^{13}C,^{18}O-containing glucose 6-phosphate in the final mixture (natural abundance is ~0.0111) = 0.011 75, and ^{13}k = ^{13}C isotope effect at C-1 from analogous hydrolysis experiments with natural abundance glucose 6-phosphate where both residual glucose 6-phosphate and glucose product were analyzed. The measured value was 1.002 ± 0.002; we have assumed this to be unity.

(10) Hermes, J. D.; Morrical, S. W.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5479.

(11) Knight, W. B.; Weiss, P. M.; Cleland, W. W., preceding article in this issue.

(12) Calculations were carried out with the BEBOVIB-IV program of Sims.[13] The reactant model was a phosphate group with a carbon attached, and the transition-state model was a symmetrical trigonal bipyramid, since K_{eq} is near unity for glucose 6-phosphate hydroiysis, if the concentration of water is expressed in the same units as the other reactants. Force constants for single bonds were assumed to be 4.5 mdyn/Å for P–O stretching and 1.9 mdyn Å/radian^2 for O–P–O bending, with 16% off-diagonal coupling between P–O stretches and 23% off-diagonal coupling between bends sharing a common bond, since these values correctly reproduce the Raman frequencies for PO_4^{3-} (Kohlrausch, K. W. F. *Ramanspectren*, reprinted by Edwards: Ann Arbor, MI, 1945. Hanwick, T. J.; Hoffman, P. *J. Chem. Phys.* **1949**, *17*, 1166). The program adjusts these force constants for changes in bond order and bond angles. In the transition-state model, the axial P–O stretches were coupled in off-diagonal position by a factor of 1.1 so that the asymmetric stretching vibration became the reaction coordinate motion with a negative frequency. These calculations should be reliable for axial bond orders of 0.01 or above, but lower axial bond orders reduce the frequency of the symmetrical out of plane wag below a value that is reasonable for the out of plane wag in metaphosphate (i.e., below 200 cm^{-1}). The frequencies of the other three vibrational modes of a trigonal PO_3 unit approach asymptotic limits as the axial bond order is reduced to zero in our transition-state model.

(13) Sims, L. B.; Burton, G.; Lewis, D. E. BEBOVIB-IV, Program No. 337, Quantum Chemistry Exchange Program, Department of Chemistry, Indiana University, Bloomington, IN 47401.

Stereochemistry of the Terminating Methyl → Methylene Elimination in Kaurene Biosynthesis

Robert M. Coates,* Stacie Canan Koch, and Shridhar Hegde

Department of Chemistry, University of Illinois Urbana, Illinois 61801

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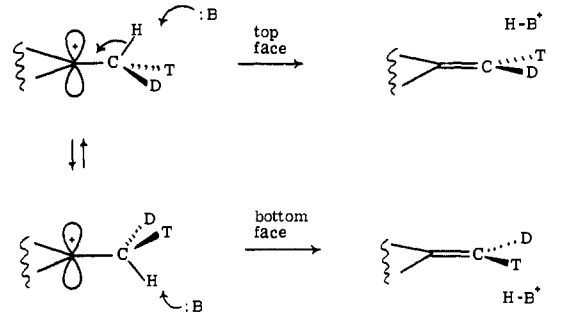
The exocyclic methylene groups commonly found in naturally occurring terpenes presumably arise by regiospecific methyl → methylene eliminations that terminate enzyme-catalyzed cyclizations. There is also a stereochemical option associated with these eliminations specified by the position of the basic group within the active site of the cyclase. The stereospecificity of the methyl → methylene elimination may be elucidated by use of chiral methyl groups.[1] We disclose herein results[2] that establish the stereospecificity of the terminating elimination in the cyclization of geranylgeranyl pyrophosphate (**2**) to kaurene (**4**) catalyzed by an enzyme extract from *Marah macrocarpus* seeds.[3]

Stereochemical Options for the Methyl/Methylene Transformation



Reduction of (*R*)- and (*S*)-β-deuteriostyrene oxide[4] with lithium triethylborotritide[5] (THF, 25 °C, 2 h) afforded (1*S*,2*R*)- and (1*R*,2*S*)-[2-^{2}H,^{3}H]-1-phenylethanol (61 and 70 mCi; 58 and 64 mCi/mmol). Successive oxidation of diluted portions with chromic acid and trifluoroperoxyacetic acid gave phenyl acetate (14–15 mCi, 71–76%) which was saponified to (*R*)- and (*S*)-sodium

(1) (a) Floss, H. G.; Tsai, M.-D.; Woodward, R. W. *Top. Stereochem.* **1984**, *15*, 253-321. (b) Floss, H. G.; Tsai, M.-D. *Adv. Enzymol.* **1979**, *50*, 243-302.

(2) Presented in part of the Second International Symposium on Synthesis and Applications of Isotopically Labelled Compounds, Kansas City, MO, Sept 5, 1985.

(3) (a) Upper, C. D.; West, C. A. *J. Biol. Chem.* **1967**, *242*, 3285-3292. (b) Oster, M. O.; West, C. A. *Arch. Biochem. Biophys.* **1968**, *127*, 112-123. (c) Schechter, I.; West, C. A. *J. Biol. Chem.* **1969**, *244*, 3200-3209.

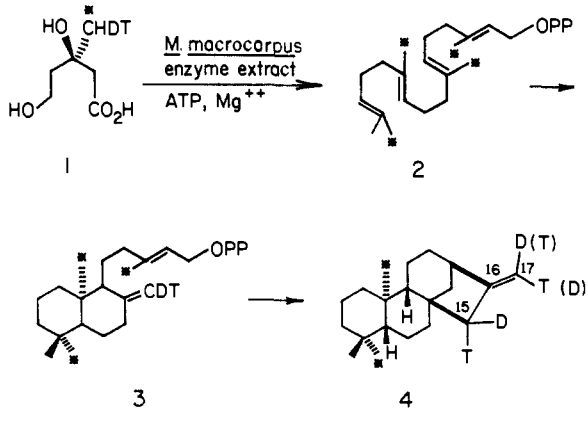
(4) (a) Preparation of (*S*)-β-deuteriostyrene oxide, $[\alpha]^{23}$ −43° (*c* 0.9, C_6H_6), optical purity ≥95% by ^{1}H NMR, from the racemate: (a) (*S*)-(1-phenylethyl)amine, 100 °C; (b) recrystallization; (c) CH_3I, $KHCO_3$, ethanol, 25 °C; (d) KOtBu, THF, reflux (30–32% overall). (b) All compounds (except high specific activity intermediates) were characterized by appropriate IR and ^{1}H NMR spectra. Satisfactory elemental analyses were obtained for new compounds by combustion or high-resolution MS.

(5) Coates, R. M.; Hegde, S.; Pearce, C. J. *J. Chem. Soc., Chem. Commun.* **1983**, 1484-1485.

Table I. Summary of Enzymatic Assays of Chiral Acetate Samples

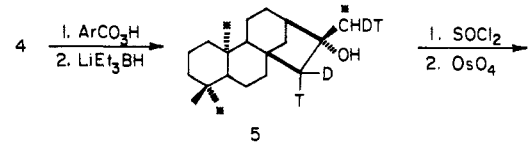
source	assays	F	ee, %	chirality
phenyl acetate	2	72.6 ± 1.4	75	*R*
phenyl acetate	4	30.5 ± 1.6	65	*S*
kaurene from (3*R*,3′*R*)-mevalonate	2	66.3 ± 2.9	54	*R*
kaurene from (3*R*,3′*S*)-mevalonate	2	42.0 ± 2.4	27	*S*

acetate.[6] Enzymatic chirality assays (Table I)[1,6-8] verified the expected configurations of the chiral methyl group but also revealed that some racemization had occurred en route (*R*, 75% ee; *S*, 65% ee).[9] Phenyl acetate was converted to (3′*R*)- and 3′*S*)-[3′,3′-^{2}H,^{3}H]mevalonolactone (1.8 and 2.8 mCi; 19–20 mCi/mmol) in four steps[10,11] via recrystallized 3-hydroxy-3-methylglutaric acid. Incubation of (3′*R*)- and (3′*S*)-mevalonate (**1**) with ATP and the S-150 enzyme extract prepared from immature *Marah macrocarpus* seeds[3,12,13] gave (19*R*,20*R*)- and (19*S*,20*S*)-[15,17,19,20-^{2}H,^{3}H]kaurene [**4**, typically 70–300 μg, 10–33% incorporation based on available (3*R*)-mevalonate) which was diluted with authentic (−)-kaurene and recrystallized 2–3 times (mp 47–48 °C, 123 and 51 μCi; 79.9 and 30.9 μCi/mmol].

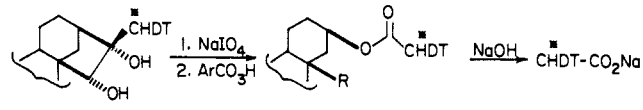


1 2 3 4

The stereochemistry of the tritium at the exocyclic methylene group was established by recreation of a chiral methyl group at C-17 and regiospecific degradation to excise chiral acetic acid. Epoxidation (*m*-$ClC_6H_4CO_3H$, $CHCl_3$ ether, 20 °C) followed by hydride reduction ($LiBEt_3H$, THF, 20 °C) afforded *exo*-kauran-16-ol [**5**, mp 213–215 °C (lit.[14] 216–217 °C)].[15] Dehydration ($SOCl_2$, pyridine, 20 °C, 30 min), chromatography on silver nitrate-impregnated silica gel to separate kaurene and isokaurene (52–62%), and hydroxylation (OsO_4, pyridine, 20 °C; aqueous $NaHSO_3$, 20 °C)[16] of the latter gave kaurene-15α,16α-diol [**6**, mp 174–176 °C (lit.[17] mp 174–175 °C), 59.6 and 27.7 μCi/mmol].



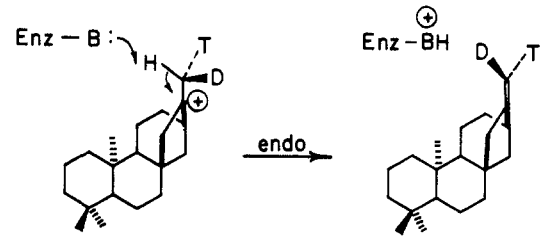
5



6 7a (R = CHO) 7b (R = OCHO)

Periodate cleavage ($NaIO_4$, 3:1 MeOH/H_2O, 20 °C, 1 h) followed by immediate Baeyer–Villiger oxidation [3,5-$(NO_2)_2C_6H_3CO_3H$, Na_2HPO_4, CH_2Cl_2, 20 °C, 2–2.5 h][18] afforded a mixture of formyl acetate **7a** (28–48%) and formate acetate **7b** (22–26%) which was saponified (NaOH, 80% aqueous MeOH). [^{2}H,^{3}H]Potassium acetate was isolated (0.49 and 0.55 μCi, 52% and 70% radiochemcial yields) from the hydrolysates by treatment with mercuric sulfate to remove formic acid,[19] steam distillation, and titration (0.1 N KOH). Addition of [^{14}C]acetate and derivatization afforded the corresponding *p*-phenylphenacyl esters which were recrystallized 3 times (^{3}H, 53.4 and 27.5 μCi/mmol; ^{3}H/^{14}C dpm ratios 6.22 and 6.17). The results of the enzymatic chirality assays on the [^{2}H,^{3}H]acetates from degradation (Table I) demonstrate overall retention of configuration, albeit with some additional loss of optical purity.[20]

The observation of retention establishes that the methyl → methylene elimination occurred with an endo orientation. If the



stereochemistry were governed by minimization of steric hindrance, stereoelectronic linkage to the preceding C-12 shift, or proton transfer to pyrophosphate anion[21] (presumably situated on the exo face),[22] an exo orientation would be predicted. Explanations based on exo shielding by pyrophosphate anion or nucleophilic participation (X-group mechanism),[23] while consistent with the observed endo elimination, must be regarded as speculative.

(6) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Eur. J. Biochem.* **1970**, *14*, 1–13. (b) Lenz, H.; Buckel, W.; Wunderwald, P.; Biederman, G.; Buschmeier, V.; Eggerer, H.; Cornforth, J. W.; Redmond, J. W.; Mallaby, R. *Ibid.* **1971**, *24*, 207–215.

(7) (a) Cornforth, J. W.; Redmund, J. W.; Eggerer, H.; Buckel, H.; Gutschow, C. *Nature (London)* **1969**, *221*, 1212–1213. (b) Luthy, J.; Retey, J.; Arigoni, D. *Ibid.* **1969**, *221*, 1213–1215.

(8) The assay procedures were adapted from those used by Profs. D. Arigoni at the ETH in Zürich and H. Floss at The Ohio State University. We are grateful to Prof. Floss for supplies of malate synthase. Authentic samples of (*R*)- and (S)-acetate provided by Prof. Floss gave F values of 80.4 ± 0.6 and 20.7 ± 1.8, respectively.

(9) Inversion in the supertritide reduction of β-deuteriostyrene oxide (like the corresponding $LiAlD_4$ and $LiBT_4$ reductions)[6] is assumed. Partial racemization may have occurred at the acetophenone stage.

(10) (a) C_3H_5MgCl, THF/ether, 0 °C, 5 h; (b) O_3, CH_2Cl_2/HOAc, −70 °C; H_2O_2, HOAc, reflux; (c) DCC, acetone, 50 °C, 3 h; (d) $NaBH_4$, *i*-PrOH, 25 °C, 24 h [12–20% overall yields of (3*R*(*S*)-mevalonate].

(11) (a) Fetizon, M.; Golfier, M.; Louis, J. M. *Tetrahedron* **1975**, *31*, 171–176. (b) Phillips, G. T.; Clifford, K. H. *Eur. J. Biochem.* **1976**, *61*, 271–286. (c) Lewer, P.; MacMillan, J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1417–1420.

(12) (a) We are grateful to Prof. C. A. West for providing the seeds and for assistance in collecting them. (b) Procedures for isolation of the enzyme extract: Hirano, S. S. Ph.D. Thesis, University of California, Los Angeles, 1976. Sherwin, P. F. Ph.D. Thesis, University of Illinois, Urbana, 1982.

(13) Mevalonate (10–20 μmol, 0.08–0.17 mM), ATP (3–6 mM), $MgCl_2$ (3–6 mM), $KHPO_4$ buffer (30–40 mM), S-150 enzyme extract (47–65% of final volume), pH 7, 30 °C, 18 h.

(14) Briggs, L. H.; Cambie, R. C.; Ruttledge, P. S. *J. Chem. Soc.* **1963**, 5374–5383.

(15) Inversion in the reduction of the exo epoxide is assumed.[9] For precedent for high exo selectivity in reactions of kaurene and related diterpenes, see: Coates, R. M.; Bertram, E. F. *J. Org. Chem.* **1971**, 2625–2631 and references cited.

(16) Baran, J. S. *J. Org. Chem.* **1960**, *25*, 257.

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(19) Friedemann, T. E. *J. Biol. Chem.* **1983**, *123*, 161–185.

(20) Some enolization of the methyl ketone intermediate could account for the somewhat larger than predicted racemization (*R*, 61% ee; *S*, 53% ee).

(21) The possibility that pyrophosphate anion might act as the base in the prenyl transferse coupling has been suggested: Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307–313.

(22) The Wenkert biogenetic pathway (a) via pimarenyl, beyeranyl, and kauranyl intermediates is usually proposed for the **3** → **4** cyclization (b). Association of pyrophosphate anion with the beyeranyl and kauranyl ions would most likely occur on the exo face. (a) Wenkert, E. *Chem. Ind. (London)* **1955**, 282–284. (b) Coates, R. M.; Cavender, P. L. *J. Am. Chem. Soc.* **1980**, *102*, 6358–6359.

(23) Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903.

Further stereochemical information is clearly needed to identify the factor(s) that determine the elimination specificity of terpene cyclases.

Acknowledgment. We are grateful to New England Nuclear for a gift of lithium tritide, to Abbott Laboratories for samples of (-)-kaurene, to Christine Klodnycky for conducting the enzymatic chirality assays, and to the National Institutes of Health for financial support (GM 13956). High-field NMR spectra were obtained with the aid of the Univeristy of Illinois NSF Regional Instrumentation Facility (NSC CHE 79-16100). The acquisition of GC/MS analyses in the School of Chemical Sciences Mass Spectrometry Center was aided by a grant from the National Institute of General Medicine Sciences (GM 27029).

Sequence-Specific Cross-Linking of Deoxyoligonucleotides via Hybridization-Triggered Alkylation

Thomas R. Webb* and Mark D. Matteucci*

Genentech, Inc.
South San Francisco, California 94080

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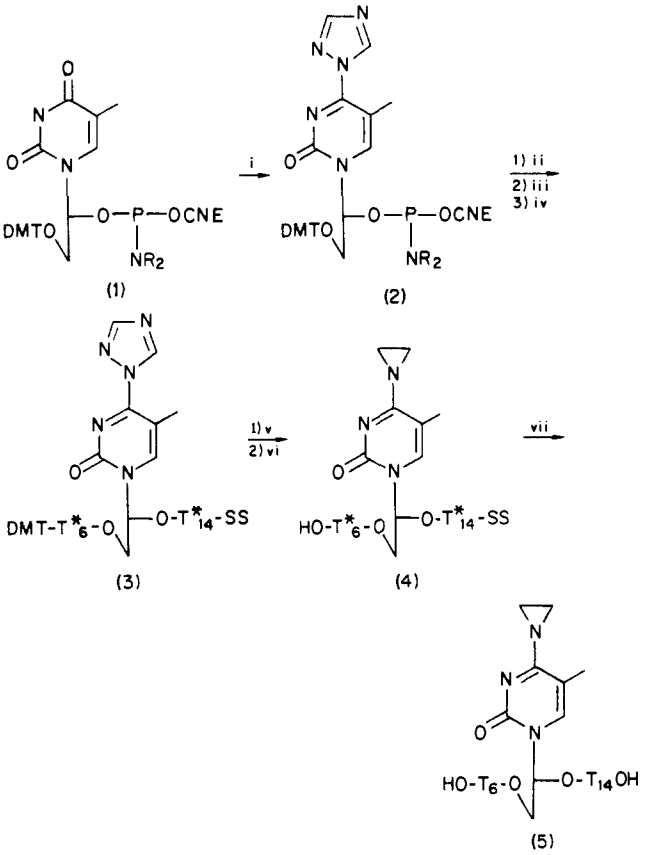
Oligonucleotide analogues that have enhanced binding to a complementary sequence are of potential biological interest. Dimer[1] and oligonucleotide[2] analogues bearing a moiety capable of intercalation show enhanced binding to complementary sequences. Oligonucleotides capable of forming a covalent cross-link with a complementary sequence in a selective manner would be the ultimate in enhanced binding. The incorporation of masked alkylating agents into DNA and RNA has been reported.[3,4] Such systems require the chemical activation of the alkylating moiety and are therefore not compatible with in vivo systems. We have developed an oligodeoxynucleotide analogue that possesses the unique property of being essentially unreactive until it is constrained in a double helix. When so constrained it forms a stable covalent bond with its complementary sequence.

We reasoned that if an analogue of one of the four bases, containing a suitably placed electrophilic center, was constrained in a Watson-Crick bonding scheme, interstrand cross-linking would result due to the proximity of a nucleophilic center on the complementary strand. We chose N^4,N^4-ethanocytosine as the electrophilic base analogue, which ought to be able to form two hydrogen bonds with a guanine base in the target oligomer. We realized that other bases such as adenine or cytosine, which possess nucleophilic amine functions, might also alkylate under these conditions.

The synthesis of deoxyoligonucleotides containing the 5-methyl-N^4,N^4-ethanocytosine (C^e) moiety required some modification of existing synthetic methods. We prepared the amidites **1** and **2** (see Scheme I) and incorporated them in a deoxyoligonucleotide by combining the methods of Huynh-Dinh et al.[5] and Koster et al.[6] (see Scheme I).

Scheme I.[a] Synthesis of the Oligomer Containing 4,4-Ethanocytosine



[a] DMT stands for 4,4'-dimethoxytrityl, CNE for 2-cyanoethyl, R for isopropyl, T_n stands for oligothymidylic acid the subscript n being the number of residues in the oligomer, likewise the * denotes CNE protecting groups on an oligothymidylic acid; SS denotes the solid support (silica gel). (i) Triazole, $POCl_3$ in acetonitrile/triethylamine; (ii) HO-T^*_{14}-SS/4-nitrotetrazole; (iii) I_2/water/lutidine; (iv) six couplings with **1** (addition of T^*_6); (v) 25% dichloroacetic acid in dichloromethane (detritylation); (vi) ethylenimine in water; (vii) concentrated NH_4OH.

Amidite **2** was prepared from **1** and used to prepare the cyanoethyl-protected oligonucleotide **3** by using the standard synthetic cycle.[7,8] The intermediate **3** (attached to silica gel support) was treated with a solution of ethylenimine[9] in water to give the protected oligomer **4**. The intermediate **4** was deprotected at phosphorus and removed from the solid support by brief treatment (~30 min, 25 °C) with concentrated NH_4OH, to yield oligomer **5**. Oligomers **6**-**10** were prepared by using methoxy-*N*,*N*-diisopropyl amidites[8] by the standard synthetic cycle[7,8] and purified by polyacrylamide gel electrophoresis (20% denaturing gel).

Oligomer **5** (containing the ethanocytidine group) was allowed to hybridize with purified oligomers **6**-**10**, which had been labeled at the 5'-end with ^{32}P using γ-^{32}P-labeled ATP and polynucleotide kinase (see Figure 1b for conditions). Analysis of these reactions by gel electrophoresis (20% denaturing gel) followed by autoradiography showed the appearance of a higher molecular weight band (Figure 1b). This band **11** was by far most prevalent in the reaction of **5** with **9**, the oligomer-bearing cytosine (Figure 1b, lane 8). A control reaction containing only labeled **5** and buffer did not show any new bands even after 7 days at 24 °C (data not shown). A band of identical mobility to **11** was observed when purified 5'-^{32}P-labeled **5** was allowed to react with unlabeled **9**, demonstrating that this new band was a product of **5** and **9** (data not shown). Product **11** is unaffected by the strongly denaturing

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