Etnangien, a Macrolide-Polyene Antibiotic from *Sorangium cellulosum* That Inhibits Nucleic Acid Polymerases

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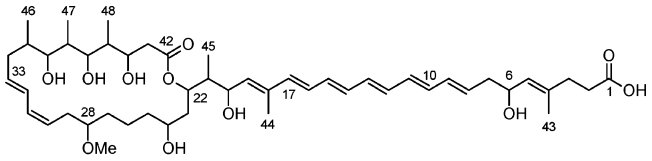
Etnangien (**1**), a new macrolide antibiotic active against Gram-positive bacteria, was isolated from the culture broth of the myxobacterium *Sorangium cellulosum*, strains So ce750 and So ce1045. Spectroscopic structure elucidation of **1** revealed a complex macrocyclic lactone bearing a modified C_{21} carboxylic acid side chain. The latter contains two allylic hydroxyl groups and an all-*E* hexaene unit, which provides the characteristic UV chromophore of **1**. Initial studies toward the mechanism of action showed that bacterial and viral nucleic acid polymerases are inhibited by etnangien (**1**).

In the course of our broad screening program for biologically active secondary metabolites from myxobacteria, many genera have been found to produce novel natural products useful as drugs or leads for further development. In particular, strains belonging to the genus *Sorangium* were found to generate fascinating structures exhibiting multiple biological activities,[1] e.g., highly potent antibiotics such as the antifungal soraphens[2] or the antibacterial sorangicins[3] and thuggacins,[4] as well as anticancer agents, e.g., the epothilones.[5]

Recently, two strains of *Sorangium cellulosum*, So ce750 and So ce1045, were discovered and found to produce a hitherto unknown antibiotic against Gram-positive bacteria. Bioactivity-guided isolation by solvent partition and chromatography provided the new macrolactone antibiotic etnangien (**1**). The name "etnangien" was created from the volcano mount "Etna", where an earth sample with one of the producing strains was collected, from the genus Sor"angi"um, and from hexa"en" (German spelling). Herein, we report the isolation, spectroscopic structure elucidation, and results of preliminary biological studies on **1**.



Etnangien (**1**)

For the production of etnangien (**1**), preferentially strain So ce1045 was cultivated in fermentors of 65 or 280 L in a complex medium containing glucose, starch, peptone from soy meal, and salts. The cultivation was performed in the presence of 1% (w/v) of the neutral resin Amberlite XAD 16, which removed the metabolites from the broth during cultivation, resulting in an average production of 5 mg/L.

Etnangien (**1**) was isolated as a yellow resin after extraction, solvent partitioning, and reversed-phase (RP) chromatography. Negative FABMS of **1** showed the molecular ion $[M - H]^-$ at *m*/*z* 839 and high-resolution FABMS revealed the molecular formula $C_{49}H_{76}O_{11}$, in accord with the ^{13}C NMR and DEPT spectra. Twelve double-bond equivalents were implied by the formula and, together with the yellow color and the UV spectrum showing four bands from 323 to 376 nm, suggested a hexaene structural element for etnangien (**1**).

Well-resolved NMR spectra of **1** were obtained in acetone-d_6. Signals for each carbon atom were detected in the ^{13}C NMR spectrum, with their multiplicities sorted by the DEPT spectrum and then correlated with the signals of their directly bound protons from $^1H-^{13}C$ HMQC NMR data (Table 1). From their distinct correlation signals in the $^1H-^1H$ COSY NMR spectrum five of the seven remaining heteroatom-bound protons were assigned as OH protons to their respective oxymethine carbons (C-6, -20, -36, -38, and -40). H/D exchange with methanol-d_4 removed the OH signals and caused solvent shifts especially among the methyl group signals.

The $^1H-^1H$ COSY NMR spectrum provided two main structural parts A and B (Figure 1) for **1**; when tracing the correlation signals from methylene H_2-41 at δ_H 2.4, a continuous sequence of signals could be followed within structural part A to a group of three aliphatic methylene groups around δ_H 1.31 to 1.46 (C-25 to C-27). Since their overlapping multiplets showed only a single further correlation, part A could be extended further immediately, following the direct correlations of H-24 to H-19, and finally onward via a COSY long-range correlation to the methyl group H_3-44. The low-field acylation shift of the oxymethine H-22 (δ_H 5.44) and the $^1H-^{13}C$ HMBC signals of the carboxyl signal C-42 (δ_C 173.6) with H-22 (besides correlations with H-41, H-40) indicated the lactone ring closure within structural part A. As a result of mutual HMBC signals, the only methoxyl group was assigned to the C-28 oxymethine.

The structure elucidation of part B originated from the olefin proton H-5 (δ_H 5.24) in two directions. According to its weak long-range correlations in the COSY spectrum the Δ^4 double bond was found to be substituted by the methyl H_3-43 group (δ_H 1.66) and the methylene H_2-3 group (δ_H 2.27). The latter has only one strong COSY correlation signal with the methylene H_2-2 group (δ_H 2.38), which carries the second carboxyl group (C-1), as indicated by $^1H-^{13}C$ correlation signals in the HMBC NMR spectrum between the carboxyl signal C-1 (δ_C 174.17) and H-2 and H-3. A correlation with the acid proton, the only H/D exchangeable proton remaining from the molecular formula, was not observed. These signals are often too broad for detection in 1D ^1H NMR spectra.

The chain of part B could be extended in the opposite direction through the observation of strong vicinal COSY correlation signals from H-5 to the AB system of two olefin protons, H-10 and H-11 (δ_H 6.24 and 6.25). These exhibited no other distinct correlations in the COSY spectrum besides overlapping signals with the remaining multiplet of six olefin carbons (C-12* to C-17* around δ_H 6.35). The position of H-17 within the multiplet was detected in the *J*-resolved ^1H NMR spectrum from its doublet signal at δ_H

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Table 1. NMR Spectroscopic Data of Etnangien (**1**) in Acetone-d_6 (^{1}H 600 MHz; ^{13}C 100 MHz)

H	δ_H	mult.	*J* [Hz]	δ_C	mult.[a]	HMBC[b]
1				174.2	s	H-3, H-2
2	2.38	m		33.0	t	H-3[c]
3	2.27	m		35.2	t	H-43, H-2, H-5
4				135.9	s	H-43, H-3 > H-2
5	5.24	dq	8.5, 1.3	129.9	d	H-43, H-7a, H-3 + H-7b
6	4.37	dt	8.3, 6.4	68.5	d	H-7a/b, H-8
7[a]	2.33	m		42.4	t	H-9, H-8 > H-6, H-5
7[b]	2.22	m				
8	5.76	dt	15, 7.6	132.6	d	H-10 or -11, H-6, H-7a/b
9	6.16	dd	10.2, 15	133.5	d	*f*
10	6.25	m		132.1	d	H-9
11	6.24	m		134.3	d	H-8, H-12[g]
12[g]	6.31	m		134.1	d	H-10 or −11 > H-13[g]
13[g]	6.35	m		134.0	d	[g]
14[g]	6.35	m		133.9	d	[g]
15[g]	6.35	m		133.6	d	[g]
16[g]	6.31	m		129.5	d	[g]
17	6.33	d	18	138.3	d	H-19, H-44, H-16[g]
18				136.1	s	H-20,H-44
19	5.53	dd	9, 1 br	135.5	d	H-44, H-20
20	4.39	dd	9, 8.1	69.6	d	H-45, H-21
21	1.98	m		43.8	d	H-45, H-20, H-19 (H-22)
22	5.44	dt	7.6, 4.6	73.5	d	H-45, H-20, H-21, H-23
23	1.65	m		38.7	t	H-22, H-25/-26/-27, H-21
24	3.51	tt	8.3, 4.1	67.9	d	H-23, H-22, H-25/-26/-27
25[a]	1.42	m		39.2	t	H-23, H-26/-27
25[b]	1.31	m				
26[a]	1.46	m		22.1	t	H-28, H-25/-27
26[b]	1.31	m				
27	1.41	m		33.2	t	H-29a/b[d]
28	3.25	ddt	4.5, 5.6, 5.9	81.1	d	OMe, H-29a/b, H-27
29[a]	2.44	m		30.7	t	H-31, H-30, H-25/-26/ -27
29[b]	2.37	m				
30	5.35	dt	10.7, 7.7 br	126.0	d	H-32, H-28, H-29a/b
31	6.06	t	11	130.9	d	H-32, H-33, H-29a/b
32	6.36	m		128.3	d	H-34b, H-31, H-30
33	5.71	ddd	6.0, 8.3, 14.8	133.3	d	H-34a, H-7a/b, H-31
34[a]	2.23	m		37.6	t	H-46, H-33, H-32, H-36
34[b]	1.95	m				
35	1.75	m		36.5	d	H-46, H-21, H-36
36	3.44	dd	7.2, 3.8	79.3	d	H-48, H-46, H-38 > H-34b/a, H-37
37	1.84	m		37.3	d	H-48, H-36, H-38
38	3.61	dd	7.2, 3.2	76.9	d	H-47, H-48, H-39, H-36, H-40
39	1.87	m		42.8	d	H-47, H-41 > H-40 > H-38
40	4.17	dt	5.4, 6.2	69.0	d	H-47, H-41, H-39
41	2.40	m	(2H)	38.7	t	H-40, H-39
42				173.6	s	H-41, H-40, H-22
43	1.66	d	1.1 NOE: H-6	16.7	q	H-3, H-5
44	1.82	d	0.7 NOE: H-20	13.2	q	H-19 > H-17
45	0.84	d	7.2	11.1	q	H-20, H-22, H-21
46	0.92	d	6.8	15.1	q	H-36, H-34a/b
47	0.98	d	6.8	7.2	q	H-38, H-36 > H-37
48	0.97	d	6.8	10.9	q	H-39, H-38, H-40
49	3.29	s		56.4	d	H-28
OH-6	3.56	m				
OH-20	3.84	d	2.6 br			
OH-36	3.73	m				
OH-38	3.89	m				
OH-40	3.98	d	3.3 br			

[a] From ^{13}C DEPT NMR spectrum. [b]HMBC correlations from proton(s) to correlated carbon; > denotes lower abundance of following signal(s). [c]Interchangeable with C-27. [d]Interchangeable with C-2. [f]Overlapping with signals marked by *g* and with C-33. [g]Assignments interchangeable due to signal overlap.

6.33 (J = 18 Hz). Exactly at that chemical shift value a proton of the polyene multiplet was correlated in the HMQC spectrum to the methine carbon signal with the highest shift (δ_C 138.3). The latter was independently specified as the C-17 signal due to strong correlations in the HMBC spectrum with H-19 and methyl group H_3-44. In this manner the connectivity of structural parts A and B of **1** was established.

In part, the configuration of the double bonds of **1** could be derived from coupling constants, with the *E* configuration indicating vicinal coupling constants of about 15 Hz being observed for $J_{8,9}$ and $J_{32,33}$. The *Z* configuration of the $\Delta^{30,31}$ double bond followed from a vicinal coupling $J_{30,31}$ of 11 Hz. The configuration of both methyl-substituted double bonds ($\Delta^{4,5}$ and $\Delta^{18,19}$) was evident from their correlations in the ROESY NMR spectrum: first, the methyl protons H_3-43 correlated with H-6, H-3, and H-2 on one side, and H-3 correlated with H-5 on the opposite side, and second, the methyl protons H_3-44 correlated with H-20 and at least one of the hexaene protons, while H-19 correlated with a proton at δ_H 6.33, which was previously shown to be H-17.

The all-*E* configuration of the remaining double bonds of the hexaene unit of **1** was suggested by the UV spectrum with bands at 323, 339, 356, and 376 nm and indirectly deduced from the nearly

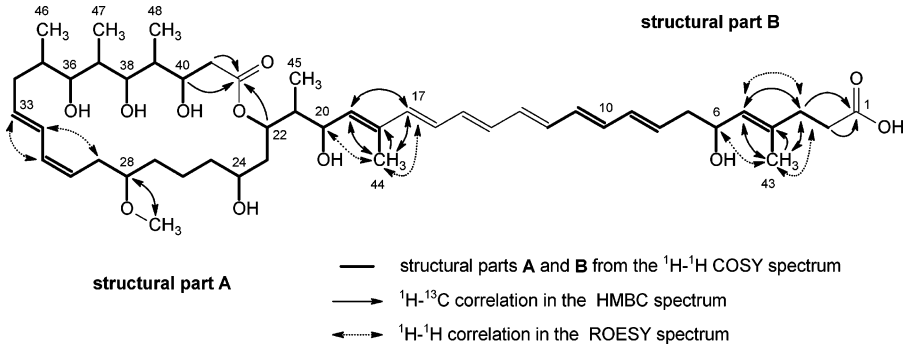

Figure 1. Structural elements of etnangien (**1**) from NMR spectroscopy.

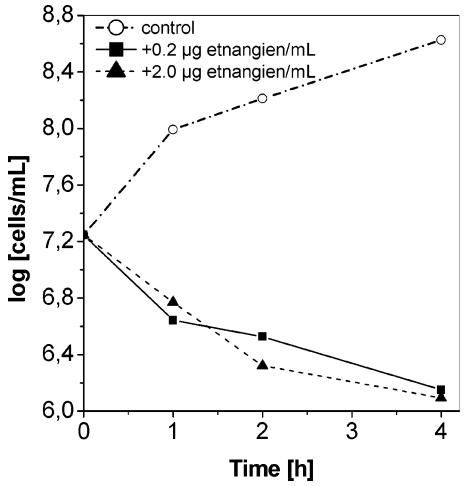

Figure 2. Effect of etnangien (**1**) on the viability of *M. luteus*. The cells were suspended in nutrient broth and incubated at 30 °C. At $t = 0$ h the culture was divided and **1** or methanol was added. At different times aliquots were diluted and spread on agar plates. After 24 h colonies were counted.

complete overlap of their ^{1}H NMR signals, while a dispersion of their ^{1}H NMR signals would be expected if one double bond were in the *Z* configuration.[6] Although part of the lactone ring, the 30*Z* and 32*E* double bonds of the diene adopted an *s-trans* spatial arrangement ($J_{31,32} = 11$ Hz), as indicated by the ROESY signals between H-29 and H-32 and H-31 and H-33.

The biological activities of **1** were tested against different microorganisms and a murine cell line. Table 2 gives selected MIC and toxicity values.

Some bacteria belonging to the Corynebacterineae, such as *Nocardia corallina* and some *Mycobacteria*, were found to be particularly sensitive to **1**, while *E. coli* and yeasts proved to be rather resistant. The high IC_{50} value observed with mouse fibroblast cells indicates that **1** may be well tolerated by cell cultures.

For further characterization of the antibiotic action of etnangien (**1**), *Micrococcus luteus* was selected. Figure 2 presents the influence of two concentrations of **1** on the number of viable cells in nutrient broth. Within 4 h the number of viable cells decreased to about 10% of the initial number. This bactericidal effect was independent of whether 0.2 μg/mL, a concentration about 3 times above the MIC, or a 30-fold concentration (2 μg/mL) was applied. Measurement of DNA, RNA, and protein synthesis showed that the formation of these macromolecules was inhibited in a similar manner (data not shown).

Purified RNA and DNA polymerases from *E. coli* (EcRNAP and EcDNAP) were inhibited by **1** with comparable dose−effect curves (Figure 3). The maximum of inhibition was in the range 50 to 60 μg/mL, although the Gram-negative *E. coli* itself was resistant to this antibiotic.

The inhibitory effect was not restricted to bacterial polymerases. The reverse transcriptase of HIV I (HIVRT) was inhibited with a

Table 2. MIC and IC_{50} Values of Etnangien (**1**)

test organism	MIC (μg/mL)
Staphylococcus aureus	1
Streptococcus faecalis	2
Bacillus subtilis	10
Bacillus megaterium	20
Micrococcus luteus	0.06
Nocardia corallina	0.12
Corynebacterium mediolanum	0.06
C. glutamicum DSM 20300	0.03
Mycobacterium phlei	0.12
M. diernhoferii DSM 43218	0.12
M. smegmatis DSM 43856	1
Escherichia coli	>80
Saccharomyces cerevisiae	>80
Rhodotorula glutinis	>80
	IC_{50} (μg/mL)
mouse fibroblast cells L929	74

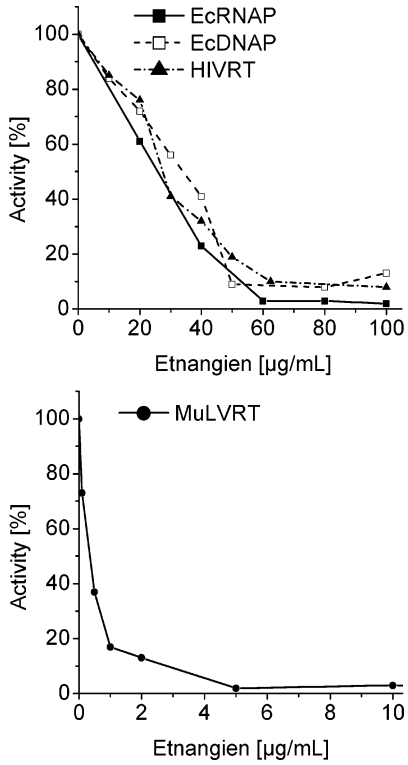

Figure 3. Dose−effect curves of the inhibition of nucleic acid polymerases with etnangien (**1**).

comparable dose−effect curve. However, the reverse transcriptase of the Moloney murine leukemia virus (MuLVRT) proved to be the most sensitive, with a maximum inhibition of 5 μg/mL.

Although nucleic acid polymerases from eukaryotes and Gram-negative bacteria are sensitive targets of etnangien (**1**), the observed

toxicity for whole cells was low (Table 2). This observation may be explained by a penetration barrier for **1** in cell culture and also in Gram-negative bacteria. However, the potent MIC values against mycobacteria make etnangien (**1**) an interesting molecule as a lead for drug development and for further biological studies to explore the mode of action more deeply. Chemical studies of the absolute configuration and structure−activity relationship should also be conducted.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a model 241 polarimeter (Perkin-Elmer), using a cuvette with a length of 1 dm. UV spectra were recorded on a U-3200 (Hitachi) UV spectral photometer with methanol as solvent. IR spectra were run on a FT-IR 20 DXB spectrometer (Nicolet). For NMR spectroscopy AM-600 and AM-300 spectrometers (Bruker) were used (^{1}H: 600 MHz, ^{13}C: 150 and 75 MHz, internal standard TMS). FABMS spectra were recorded on a MAT 95 mass spectrometer (Finnigan) [resolution $M/\Delta M = 1000$; high resolution $M/\Delta M = 10\,000$]. Analytical TLC was performed with TLC aluminum sheets [silica gel Si 60 F_{254}, 0.25 mm (Merck)]. For detection the UV absorption at $\lambda = 254$ nm was observed. Columns of 4 × 250 mm with Nucleosil RP-18-7-100 (Macherey-Nagel) were used for analytical HPLC. All operations with etnangien-containing material were done under dimmed light or in brown glassware.

Cultivation of *Sorangium cellulosum*, Strain So ce1045. The strain was cultivated at 30 °C in a medium containing soluble starch, 0.25%; insoluble starch (Cerestar), 0.25%; peptone from soy meal, 0.3%; soy meal, defatted, 0.05%; $CaCl_2 \times 2\ H_2O$, 0.1%; and Na-Fe-EDTA, 8 mg/L. The pH was adjusted to 7.6 before autoclaving. Glucose × H_2O, 0.3%, and $MgSO_4 \times 7\ H_2O$, 0.1%, were autoclaved at a 100-fold concentration and added separately. With an inoculum of 1.5%, a stirring rate of 80/min, and an aeration of 0.5 L/L medium × min the pO_2 decreased from 100% to 10% within 7 days. Fermentations were run in the presence of 1% Amberlite XAD 16 (Rohm and Haas) and ended after 8 days, 1 day after starch and glucose were used up. Then the XAD was recovered from the culture by sieving. In fermentors the pH was kept between 7.0 and 7.8 by addition of KOH and H_2SO_4, respectively. For cultures in Erlenmeyer flasks the medium contained HEPES buffer pH 7 (1%).

Extraction and Isolation. Amberlite XAD-16 and cells (2.8 kg) from a 280 L fermentation batch of *S. cellulosum*, strain So ce1045, were thoroughly rinsed with water to remove adherent cells and then transferred to an open chromatography column (10 × 30 cm). After elution with four bed volumes of methanol (4 × 2.2 L) the eluate was buffered with 0.1 N NH_4 acetate solution (50 mL) and concentrated *in vacuo* (40 °C water bath) until an oil−water mixture had formed (2 L). This mixture was extracted four times with EtOAc. The combined organic layers were evaporated with toluene (200 mL) and concentrated *in vacuo* to yield a brown, oily residue. This was dissolved in 300 mL of ice-cold EtOAc and extracted four times with 100 mL of cold 2.5% sodium hydrogen carbonate solution (pH 8). The separation of the layers was achieved by centrifugation at 0 °C. After evaporation the EtOAc layer yielded 17.7 g of enriched neutral byproducts.

The combined cold aqueous carbonate layers were acidified to pH 5 by ice-cold dilute acetic acid and extracted four times with 250 mL of cold EtOAc. The combined EtOAc portions were partitioned once with cold 5% ammonium acetate (pH shift to 6.8) before they were evaporated. Evaporation was finished in high vacuum after addition of toluene to yield 14 g of an dark, oily product. For intermediate storage, 100 mg of 1,2-dihydroxy-4-*tert*-butylbenzene was added as stabilizer to a solution of this material in 200 mL of methanol and kept at −70 °C.

About 1 g of the raw product in 2.5 mL of methanol was injected for preparative MPLC (column 480 × 30 mm, ODS-AQ 120 A, 16 μm (YMC); solvent A: 50% methanol, solvent B: 100% methanol (each 0.05 M ammonium acetate), gradient: 35% B (for 15 min, rising to 55% B in 120 min, 55% B for 40 min; flow 18 mL/min; detection UV absorption at 365 nm); the main peak eluting at 62 min was collected. Evaporation yielded an oily, aqueous buffer mixture, which was extracted with three portions of EtOAc. After re-extraction with a small portion of water, the solvent was completely removed by evaporation to yield 113 mg of pure etnangien (**1**) as a yellow residue. The compound was found to be soluble in lower alcohols, ethyl acetate, and dichloromethane and slightly in water (depending on the pH value). Compound **1** was unstable in solution at room temperature and decomposed after a few days. The stability could be extended to a few weeks using tanned (brown) sample flasks and vials, oxygen-free solvents, and a nitrogen atmosphere at −70 °C. Analytical HPLC: column 4 × 250 mm, Nucleosil-100 RP18, 7 μm; methanol−water, 75:25, 0.01 M phosphate buffer, 1.5 mL/min, UV detection 355 nm. $t_R = 4.6$ min. TLC: TLC aluminum sheets silica gel 60 F_{254} (Merck); solvent CH_2Cl_2−methanol, 8:2. Detection: UV adsorption at 254 and 366 nm. Spraying with vanillin−sulfuric acid followed by heating to 120 °C gave brown spots. $R_f = 0.41$.

Etnangien (1): $[\alpha]_D^{21}$ +18.7 (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ϵ) 216 (4.24), 235 (4.23), 323 (4.46), 339 (4.78), 356 (5.00), 376 (5.02) nm; IR (KBr) ν_{max} 3412 (s), 3017 (s), 2967 s), 2931 (s), 1714 (s), 1458 (m), 1439 (m), 1383 (m), 1284 (m), 1181 (m), 1094 (s), 1041 (m), 1004 (s), 986 (w) cm^{-1}; NMR data, see Table 1; (−)-HRFABMS *m*/*z* 839.5324 $(M - H)^-$ (100%) (calcd for $(C_{49}H_{76}O_{11} - H)^-$ *m*/*z* 839.5309).

Biological Testing. The *S. cellulosum* strains were isolated from soil samples. MIC values were determined by serial dilution.[3b] Viability of *Micrococcus luteus* was measured according to a literature procedure.[7] Dose−response curves for nucleic acid polymerases (Roche, formerly Boehringer Mannheim) were done according to published procedures.[8,9] Test conditions for all enzymes were as follows: volume 200 μL, temperature 37 °C; stop of the reaction by addition of cold 7% perchlorid acid (2 mL). The templates and precursors were as follows: DNA polymerase I (*E. coli*): 0.5 units of enzyme, 0.25 A_{260}-units of poly d(A-T) and dATP/TTP (with deoxy[1′,2′,2,8-^{3}H]-adenosine 5′-triphosphate; specific activity 86 Ci/mmol, Amersham), incubation time 30 min; RNA polymerase (*E. coli*): 0.5 units of enzyme, 0.25 A_{260}-units of poly d(A-T) and ATP/UTP (with [5,6-^{3}H]-uridine 5′-triphosphate sodium salt; specific activity 35−50 Ci/mmol, NEN), incubation time 15 min; reverse transcriptase from HIV I (0.5 units) and M-MuLV (1 unit): 0.125 units of poly (A).$(dT)_{15}$ and methyl-[1′,2′-^{3}H]-thymidine 5′-triphosphate (specific activity 116 Ci/mmol, Amersham), incubation time 40 min.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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