Inhibitory effect of flavonoids on DNA-dependent DNA and RNA polymerases

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Received 9 May 1988; accepted 12 July 1988

Summary. Flavonoids, (−)-epigallocatechin (**1**), myricetin (**2**) and quercetin (**3**), were investigated for inhibitory effects on *E. coli* DNA polymerase I and T7 bacteriophage RNA polymerase. In both DNA and RNA synthesis, **1** and **3** inhibited enzyme reactions by non-competitive and mixed type inhibition respectively, with regard to template DNAs. Myricetin (**2**) inhibited DNA and RNA polymerase reactions by mixed type and competitive type inhibition, respectively, with template DNAs. It was suggested that **2** interacts with covalently closed circular DNA.
Key words. Flavonoids; DNA polymerase; RNA polymerase; inhibitory effect.

For plant flavonoids, many pharmacological activities have been described[1]. Recently, a flavonoid, (−)-epigallocatechin (**1**) (for structure, see fig. 1), was isolated as an antibacterial and cytotoxic constituent from the Okinawan medicinal plant *Elaeagnus glabra* (Elaeagnaceae)[2–4]. This flavonoid has been shown to inhibit DNA synthesis in *Proteus vulgaris* cells[3] and RNA synthesis in *Staphylococcus aureus*[3] and HeLa cells[4]. In our previous work[3,4] myricetin (**2**) had also been shown to inhibit DNA or RNA synthesis in these bacterial and carcinoma cells.

The present work was undertaken to investigate the inhibitory effect of **1** and **2**, and also of the pharmacologically important flavonoid, quercetin (**3**)[1], on DNA-dependent DNA and RNA polymerases.

Materials. (−)-Epigallocatechin (**1**) was isolated from *E. glabra*, and **2** and **3** were purchased from Laboratory Sarget. *Escherichia coli* DNA polymerase I and T7 bacteriophage RNA polymerase were obtained from Boehringer and Toyobo, respectively. As template DNA, calf thymus DNA (200 bases with nick) obtained from Sigma was used for the DNA polymerase, and pGEM-1 (2.9 kb cyclic plasmid containing T7 promoter) (cyclic form) from Promega Biotec. for RNA polymerase. [α-^{32}P]dCTP (1.11×10^{14} Bq/mmol) and [α-^{32}P]GTP (1.48×10^{13} Bq/mmol) were obtained from Amersham. Unlabeled dNTPs (dATP, dGTP, dTTP and dCTP) and the corresponding NTPs were from Sigma. RNase inhibitor (RNasin) was from Takara Shuzo.

Methods. The mixture for the DNA polymerase reaction was prepared according to the published method[5]. A total volume of 20 µl of a mixture contained 67 mM KPO_4 (pH 7.4), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 67 µM each of dATP, dGTP and dTTP, 6.7 µM dCTP, 0.084 µM [α-^{32}P]dCTP, 0.75–12 µg activated calf thymus DNA and 0.13 units enzyme. After preincubation at 37 °C for 10 min, the reaction was initiated by adding the enzyme, and continued at 37 °C for 10 min. The reaction was stopped by heating at 95 °C for 2 min. In order to inhibit the reaction, 400 µM **1**, 4 µM **2** or 12 µM **3** in EtOH was added to the reaction mixture before the preincubation.

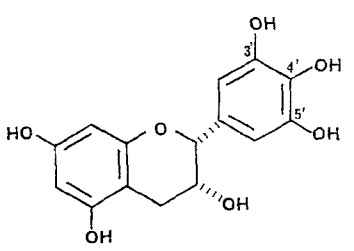
DNA polymerase activity was determined according to Schetters et al.[6]. An aliquot of the reaction mixture was chromatographed on a Whatman 3MM paper strip (2 × 23 cm) by elution with a 1:1 mixture of 1 M CH_3COONH_4 (pH 5) and EtOH. The radioactive precursor incorporated into newly formed DNA stayed at the origin, whereas unincorporated radioactive material moved to the top. From the ratio between the incorporated and unincorporated radioactivity, the amount of dCMP incorporated was calculated (in picomoles).

The assay method for RNA polymerase activity was the same as for DNA polymerase assay except for the reagents used and their amounts[7], which were as follows: 20 µl of total volume; 40 mM Tris-HCl (pH 7.6); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 20 units RNasin; 0.1 mM each ATP, GTP, CTP and UTP; 0.63 µM [α-^{32}P]GTP; 0.02–0.32 µg pGEM-1; and 6.25 units enzyme; flavonoids: 2 mM **1**; 5 µM **2**; and 5 µM **3**.

Interaction of the flavonoids with covalently closed circular pGEM-1 (ccc pGEM-1) was examined by incubation of a flavonoid (1, 10, 100 and 1000 µM) or ethidium bromide (0.0013, 0.013, 0.13 and 1.3 µM) with ccc pGEM-1 (0.5 µg) in 40 mM Tris-HCl (pH 7.6) (10 µl) at 37 °C for 20 min. The reactants were separated by electrophoresis on 1.5% agarose gel (Sigma) with Trisborate buffer. ccc pGEM-1 and Bam H1 (restriction enzyme, Toyobo)-treated pGEM-1 (linear form) were used as the references.

Results and discussion. As shown in figure 2, both DNA (A) and RNA polymerase (B) reactions proceeded almost linearly during the first 15 min after addition of the enzymes. Therefore, a 10-min incubation was employed in subsequent experiments. The inhibitory effect of the flavonoids on DNA and RNA polymerase reactions was determined as a function of the amount of template DNAs (fig. 3). The amounts and concentrations of the template DNAs and the flavonoids are shown in the legend of figure 3. All the flavonoids inhibited both DNA and RNA polymerase reactions, showing 25–35% and 70–83% inhibition for DNA (fig. 3A) and RNA (fig. 3B) polymerase activities, respectively, as compared with the control reactions.

The results shown in figure 3 were then plotted as double-reciprocal plots (Lineweaver-Burk plots) of the incorporates dCMP and GMP (both in picomoles) versus the concen-



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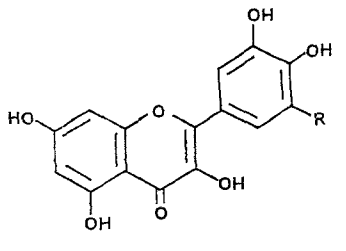

2 R=OH

3 R=H

Figure 1. Structures of flavonoids studied.

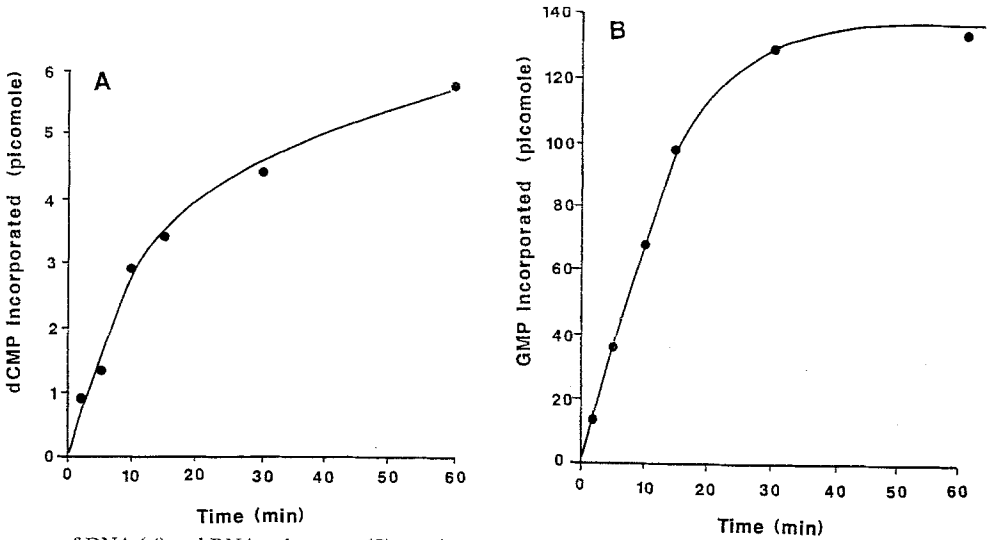

Figure 2. Time course of DNA (*A*) and RNA polymerase (*B*) reactions. The assay method is described in the text. Calf thymus DNA: 3.3 µg and pGEM-1: 0.2 µg.

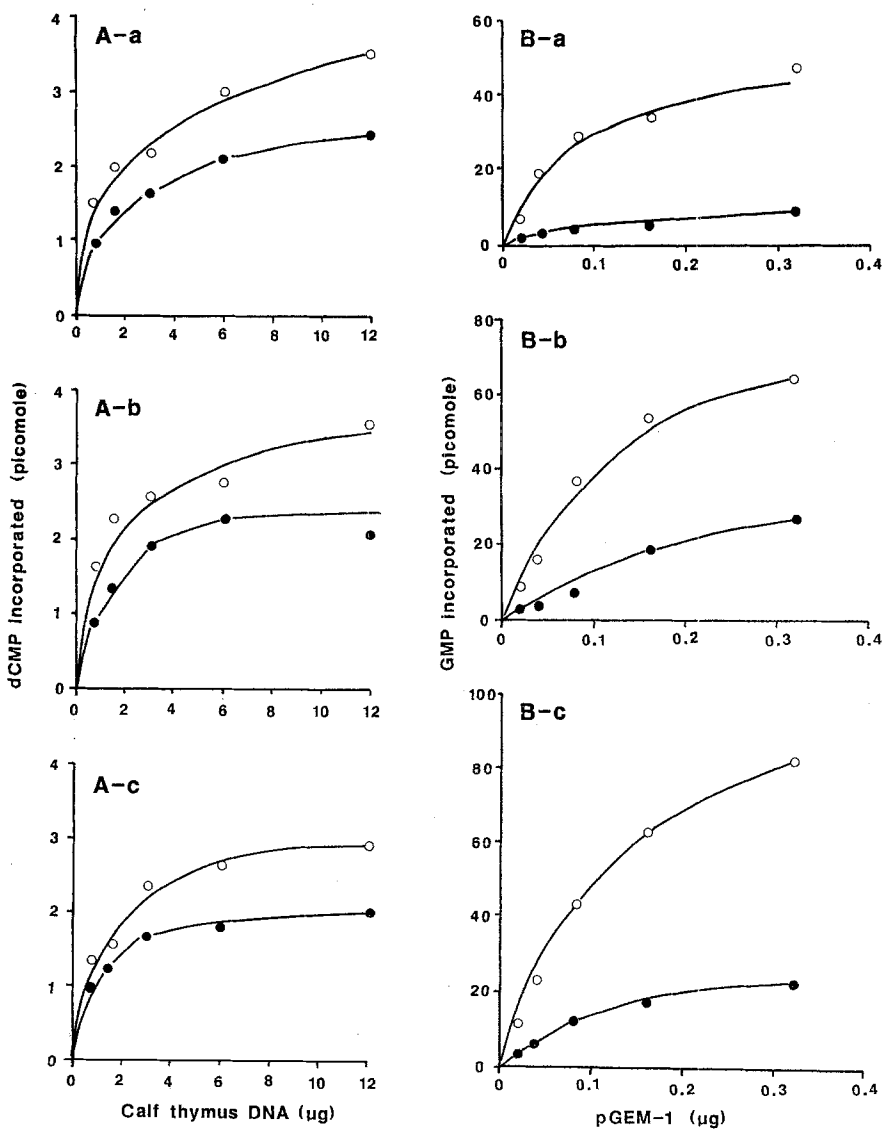

Figure 3. DNA polymerase (*A*) and RNA polymerase (*B*) reactions in the absence (–○–○–) and presence (–●–●–) of **1** (*a*), **2** (*b*) and **3** (*c*) as a function of the amount of the template DNAs [calf thymus DNA (*A*): 0.75, 1,5, 3, 6 and 12 µg; pGEM-1 (*B*): 0.02, 0.04, 0.08, 0.16 and 0.32 µg]. Concentrations of the flavonoids: **1**, 400 µM (*A-a*) and 2 mM (*B-a*); **2**, 4 µM (*A-b*) and 5 µM (*B-b*); **3**, 12 µM (*A-c*) and 5 µM (*B-c*). The assay method is described in the text.

tration of calf thymus DNA ($\times 10^{-6}$ M) and pGEM-1 ($\times 10^{-9}$ M), respectively (fig. 4A and B). The results revealed that the inhibitory actions of the flavonoids on DNA and RNA polymerases varied. In some cases inhibition with regard to the template DNA was 'competitive'; in others it was 'non-competitive' or of the 'mixed' type. From the plots. *Km* and *Ki* values were calculated as indicated in figure 4. From the *Ki* values obtained, inhibitory activity of the flavonoids was **2** > **3** ≫ **1** in both DNA and RNA polymerase reactions. The effective inhibitory concentrations for the polymerase reactions were different among the flavonoids, and these concentrations were much higher than the effective concentrations of the compounds for inhibition of DNA synthesis in HeLa cells (about 0.09 μM/ml)[4]. This implies that the flavonoids have other modes of inhibitory action on DNA and RNA synthesis besides polymerase inhibition.

From figure 4A-a and 4B-a, **1** was shown to inhibit DNA and RNA polymerase reactions non-competitively with regard to the template DNAs. Quercetin (**3**) inhibited both polymerase reactions by the mixed type of inhibition (fig. 4A-c and 4B-c). In the case of **2**, it showed the mixed type of inhibition in DNA synthesis and typical competitive type inhibition in RNA synthesis (fig. 4A-b and 4B-b, respectively). From these results, **1** is assumed to inhibit DNA and RNA synthesis by forming enzyme-substrate-inhibitor (ESI) complex. In **2**, the formation of such a complex is also presumed in inhibition of DNA polymerase reaction. But, **2** acts as a competitor with the substrate in binding to RNA polymerase.

Figure 5 shows the results of the experiment to investigate the relaxation effect of the flavonoids on ccc pGEM-1 (lane 1) which contained a trace amount of the open circular form of the DNA, but not linear DNA (lane 18). Of the agents tried, 1.3 μM ethidium bromide (intercalator) clearly yielded open circular DNA (lane 17). Production of open circular DNA was clearly observed with 1000 μM of **2** (lane 9). The production was very little with 100 μM of **3** (lane 13), and **1** did not produce open circular DNA even at 100 μM (lane 5). The results indicate interaction of **2** with ccc pGEM-1. Inspection using a computer graphic model has shown that **2** has a completely planar structure[3] due to the planar A/C ring system and the symmetrically substituted planar B-ring (fig. 1). This molecular planarity of **2** can be expected to increase its ability to intercalate into the strands of the template DNA, as is seen in the case of intercalation of actino-

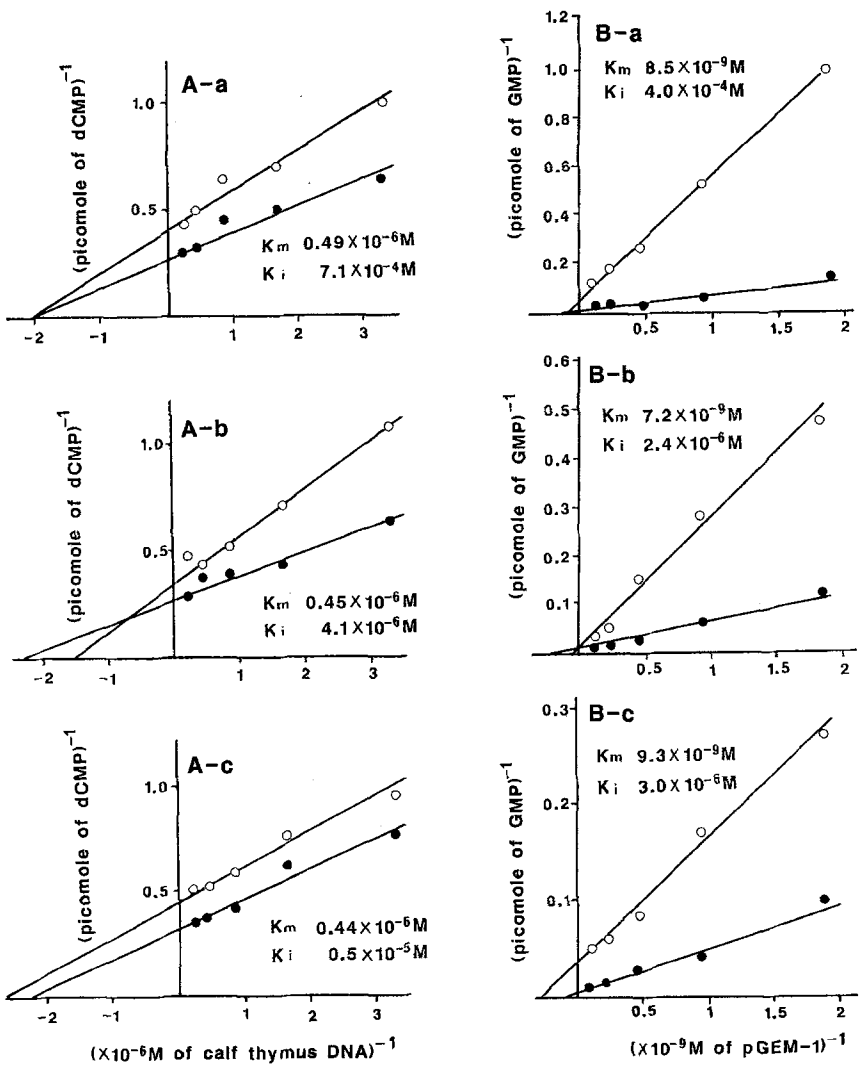

Figure 4. Double-reciprocal plots of DNA (*A*) and RNA (*B*) polymerase activities versus the amount of template DNAs in the absence (-○-○-) and presence (-●-●-) of **1** (*a*), **2** (*b*) and **3** (*c*). The amounts or concentrations of calf thymus DNA, pGEM-1 and the flavonoids are described in the legend of figure 3. *Km* and *Ki* values of the flavonoids are shown in each plot.

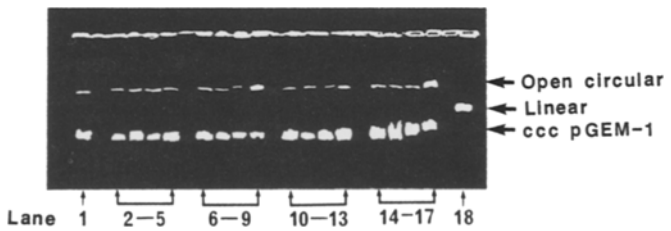

Figure 5. Interaction of **1** (lanes 2–5), **2** (lanes 6–9) and **3** (lanes 10–13) with ccc pGEM-1. Lanes 1 and 18 on the agarose gel are reference samples of ccc pGEM-1 and linear pGEM-1, respectively. Concentrations of the flavonoids: 1 μM (lanes 2, 6 and 10), 10 μM (lanes 3, 7 and 11), 100 μM (lanes 4, 8 and 12) and 1000 μM (lanes 5, 9 and 13). Effect of 0.0013, 0.013, 0.13 and 1.3 μM ethidium bromide is indicated on lanes 14–17, respectively. Experimental details are described in the text.

mycin D[8]. It has already been suggested[9] that polar flavonoids have this intercalative property. The planarity of **3** may be slightly weakened by the asymmetrically substituted B-ring (slight rotation of B-ring against the plane of the A/C ring system). Because of its nonplaner structure, **1** seems to cause no production of open circular DNA. The planarity of their structure seemed to have a correlation with the potency of flavonoids in producing open circular DNA. However, the necessary concentration (1000 μM) of the flavonoids was much higher than that (1.3 μM) of ethidium bromide.

Quite recently, (−)-epicatechin [a compound with one OH (5' position) less than **1**] has been reported to cleave the phage ϕX 174 DNA strand in the presence of Cu(II) ions[10]. It is possible that the addition of the metal ion would make the cleavage of ccc DNA by the flavonoids (**2, 3** and even **1**) possible at lower concentrations than 1000 μM.

Nose's report[11] describes the inhibitory effect of **3** on mouse RNA polymerase II activity; the transcription of naked DNA with the polymerase was strongly inhibited by mutagenic flavonoids including **3**, but weakly by non- or weakly mutagenic flavonoids. Myricetin (**2**) has also been reported to have mutagenicity[12]. In the present study, the mutagenic **2** and **3** also show a stronger inhibitory effect on the polymerase activities than the non-mutagenic **1**.

Thus, this paper is the first report on the mode of inhibitory activity of flavonoids against DNA-dependent DNA and RNA polymerases.

Acknowledgments. We thank Dr F. Hishinuma and Dr M. Itaya of our institute for their helpful discussion during the course of this work.

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0014-4754/88/100882-04$1.50 + 0.20/0

Inhibition of mushroom tyrosinase by 3-amino-L-tyrosine: Molecular probing of the active site of the enzyme

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Received 24 February 1988; accepted 24 June 1988

Summary. We report the ability of 3-amino-L-tyrosine to act as a fully reversible competitive inhibitor of mushroom tyrosinase. The inhibition is linked to the ortho-aminophenol structure, and a copper bridging mechanism in the active site is proposed.
Key words. Mushroom tyrosinase inhibition; 3-amino-L-tyrosine; ortho-aminophenols; di-copper active site.

Tyrosinase (EC 1.14.18.1) is a widespread copper-containing enzyme which catalyzes two oxidoreduction reactions involving molecular oxygen[1] and phenolic structures in the presence of reductants as shown in figure 1. The resulting unstable o-quinones typically polymerize to form pigments such as humin or melanin. However, in the presence of an excess of external reductant, the reaction will stop at the catechol level (reaction I, fig. 1); such a system has been shown to be an efficient source of o-dihydroxyphenols[2]. We report the competitive inhibition of reaction (I) by 3-amino-L-tyrosine and some structural analogs. On the basis of these results we suggest a mechanism for this inhibition using the model proposed earlier for the active site of this enzyme.

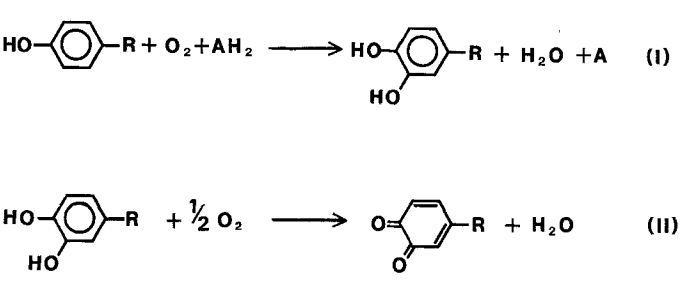

Figure 1. Mono- and di-phenoloxidase activity of tyrosinase (AH_2 = reductant).