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Communication

Effect of Synthetic Hydroxy Isothiocyanates on a Bacterial Virus and DNA

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The Effect of hydroxy isothiocyanates on a bacterial virus and M13 DNA was examined. Hydroxy-substituted phenyl and phenyl alkyl isothiocyanates, especially 2-(3,4-dihydroxyphenyl)ethyl isothiocyanate(IT-Dop) synthesized from dopamine, showed antiviral activity on ϕK. In transfection experiments with M13 mp DNA species, IT-Dop inhibited the single-stranded (SS) molecule more effectively than the double stranded replicative form (RF) DNA. These effects were dependent on reaction time, and on IT-Dop concentration. An additional experiment indicated that treatment with IT-Dop suppressed annealing (reassociation) of denatured DNA. These results indicate that IT-Dop reacts mildly with virus and SS DNA.

Key words: hydroxy isothiocyanate; 2-(3,4-dihydroxyphenyl)ethyl isothiocyanate; antiviral; DNA; transfection

Naturally occurring and synthetic isothiocyanates (ITCs) have been widely studied for their antibacterial and antifungal activities.[1,2] In a previous paper, we reported that a novel hydroxy ITC, 2-(4-hydroxyphenyl)ethyl ITC synthesized from tyramine, showed strong antifungal and antibacterial activities, and that a possible target of the ITC compounds is the cellular sulfhydryl group.[3] In addition, we found that the ITC had a concentration-dependent dual action, "antimicrobial synergism and antagonism," with aminoglycoside antibiotics such as streptomycin.[4,5]

In recent years, infectious diseases due to new types of viruses are emerging one after another, and the need for chemicals with infection-suppressive effects on viruses is well recognized. The antiviral activity of some ITCs, for example allyl ITC, benzyl ITC, horseradish extract, and so on, has been tested on herpes simplex, influenza, mouse hepatitis, human rhino virus, *etc.*[6–8] Based on these reports, we expected that hydroxy ITCs, which have distinct antimicrobial activities, might have excellent antiviral effects as well. This report is concerned with their antiviral activities and action on bacterial virus DNA.

Allyl, phenyl, and 2-phenylethyl ITC were purchased from commercial sources, and other ITCs were synthesized as previously reported.[9–11] *E. coli* W3110 and phage ϕK[12] were provided by Dr. K. Kodaira (Toyama University), and *E. coli* JM109, M13 mp18 SS and RF DNA were purchased from Takara Bio (Otsu, Japan). Each viral DNA solution was suitably diluted with 50 mM Tris–HCl buffer (pH 7.5). *E. coli* strains were cultivated at 37 °C overnight in LB composed of 10 g of trypton (Difco[BD]: NJ, USA), 5 g of yeast extract (Difco), 10 g of NaCl and 10 ml of 1 M $CaCl_2$, per liter.

The effect of ITCs on infectivity of ϕK. One hundred µl of ITCs (DMSO solution) were added to 2 ml of ϕK suspension (1.3×10^6 PFU/ml in 0.15 M NaCl). The mixture was kept at 37 °C for a defined time and diluted with DF composed of 1 g polypepton (Wako:Osaka, Japan), 3 g of NaCl and 0.1 g of $MgSO_4 \cdot 7H_2O$, per liter. To 2 ml of melted soft agar (0.7% in NB) kept at 47 °C, 0.2 ml of indicator *E. coli* W3110 culture premixed with 0.02 ml of 1 M $CaCl_2$ and 0.1 ml of diluted virus sample were added successively. After a brief incubation, soft agar was poured onto 15 ml of solid bottom agar medium (1.4% agar in LB) in a 90-mm diameter plastic dish and solidified, and the plate was incubated at 37 °C for 5 h. Infectivity was expressed in plaque-forming units (PFUs).

The effect of IT-Dop on infectious with viral DNA. To 45 µl of M13 mp18 SS or RF DNA, 5 µl of serially

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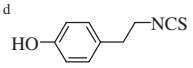
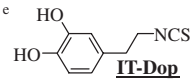
Abbreviations: IT-Dop, 2-(3,4-dihydroxyphenyl)ethyl isothiocyanate; ITC(s), isothiocyanate(s); SS, single-stranded; RF, double-stranded replicative form; PFU, plaque-forming unit

Table 1. Effect of Isothiocyanates on Infectivity of Bacterial Virus ϕK

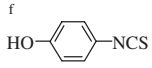
Isothiocyanate[a]	PFU/ml[b]	Relative infectivity
Unadded control[c]	6.1×10^5	
Hydroxy ITCs		
2-(4-Hydroxyphenyl)ethyl ITC[d]	4.5×10^3	7.5×10^{-3}
2-(3,4-Dihydroxyphenyl)ethyl ITC[e]	5	8.2×10^{-6}
4-Hydroxyphenyl ITC[f]	9.6×10^2	1.6×10^{-3}
trans-4-Hydroxycyclohexyl ITC	4.0×10^5	6.6×10^{-1}
6-Hydroxyhexyl ITC	2.8×10^5	4.6×10^{-1}
Other ITCs		
2-Phenylethyl ITC	3.0×10^5	4.9×10^{-1}
2-(4-Methoxyphenyl)ethyl ITC	2.6×10^5	4.3×10^{-1}
2-(4-Acetoxyphenyl)ethyl ITC	3.1×10^5	5.0×10^{-1}
Phenyl ITC	2.5×10^5	4.1×10^{-1}
6-Hexyl ITC	2.1×10^5	3.4×10^{-1}
p-Xylylene DITC(diisothiocyanate)	1.6×10^5	2.6×10^{-1}
Allyl ITC	2.9×10^5	4.8×10^{-1}

[a]ITC concentration in ϕK suspension was 4.46 µmol/ml.
[b]ϕK was treated with ITC for 24 h at 37 °C.
[c]Instead of ITC solution, only DMSO was added to ϕK suspension.

[d] 

[e] 

IT-Dop

[f] 

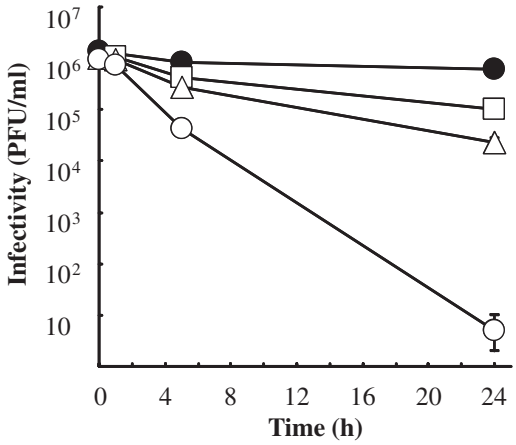


Fig. 1. Time Course of Inactivation of ϕK by Treatment with Hydroxy ITCs.

ϕK (1.3×10^6 PFU/ml) was incubated with 2.23 µmol/ml of hydroxy ITC as the indicator at 37 °C. At the indicated times, the aliquot was removed and diluted, and infectivity was assayed using *E. coli* W3110. The results of PFU measurement are the averages ± S.D. of four experiments. Hydroxy ITCs: ○, 2-(3,4-Dihydroxyphenyl)ethyl ITC; △, 4-Hydroxyphenyl ITC; □, 2-(4-Hydroxyphenyl)ethyl ITC; ●, control (without ITCs).

diluted solutions of IT-Dop in DMSO was added, and the mixtures were kept at 37 °C for a defined time. The infectivity of the DNA was determined by the $CaCl_2$ method,[13] as follows: An overnight culture of *E. coli* JM109 was diluted 10-fold with LB and incubated at 37 °C to 1.5 O.D.$_{660}$. Cells collected by centrifugation, were washed and suspended to 15 O.D.$_{660}$ with chilled 0.05 M $CaCl_2$. For the transfection assay, 0.1 ml of competent cell suspension was mixed at 0 °C with 50 µl of the DNA treated with ITCs. After it stood for 30 min at 0 °C, the mixture was heat-pulsed at 37 °C for 3 min and chilled at 0 °C for 5 min, diluted with ice-cold 0.05 M $CaCl_2$, and plated with indicator *E. coli* JM109.

The effect of various ITCs on the infectivity of free ϕK phage is shown in Table 1. It is obvious that ITCs with a hydroxy-substituted phenyl structure exhibited remarkable antiviral activities. Above all, IT-Dop with two hydroxyl groups showed the highest antiviral activity: less than 10^{-5} of the phage titer survived the treatment. On the other hand, phenylethyl ITCs without the hydroxy group caused only feeble inhibition. These results suggest that the existence of two hydroxyl groups in IT-Dop plays an important role in the antiviral effect. It appears possible that ITC with three hydroxyl groups has more potent antiviral activity. Attempts to synthesize the ITC derivative from 6-hydroxydopamine, were, however, unsuccessful, probably due to the resonance effect. Figure 1 shows the results of time course experiments on inactivation of ϕK exposed to hydroxyphenyl-substituted ITCs. Inactivation of the free virus was dependent on duration of incubation with the chemicals. A roughly logarithmic relationship was observed between infectivity loss of ϕK and the duration of exposure to IT-Dop.

In subsequent experiments, the influence of IT-Dop treatment was examined on free SS DNA of the M13 mp virus and the RF molecule (intracellular double-stranded circular DNA from virus-infected bacteria), both of which were infective to Ca^{2+}-induced competent *E. coli*. Figure 2 shows the dose response of IT-Dop on the transfectivity of these DNAs at 30 min (A) and 5 h (B). Up to 0.8 µmol/ml, the reagent did not significantly affect the RF DNA at 30 min. As to SS DNA, however, a concentration-dependent loss of infectivity clearly occurred during the incubation time. When the incubation time was prolonged to 5 h, both DNAs underwent inactivation in a dose-dependent manner, but SS was distinctly more sensitive than RF, in which a functional group(s) reactive to IT-Dop were probably protected by inter-strand hydrogen-bond formation.

In order to determine which nucleotide residue was sensitive to hydroxy ITC, four deoxyribonucleoside-5′-triphosphates were incubated successively with IT-Dop. Their UV-absorption curves were nearly identical with those of the various untreated controls, suggesting that a physicochemical change in nucleotides, if it occurred, might be very subtle. Although the bioassay using infectious viral DNAs was extremely sensitive, *E. coli* B DNA was, for reasons, used in the subsequent physicochemical experiments. The DNA (type VIII, Sigma-Aldrich:MO, USA) was heat-denatured and subjected to IT-Dop treatment, but no significant spectrophotometrical change was detected between the treated DNA and an untreated control. On the other hand, IT-Dop treatment distinctly inhibited annealing (reassociation) of the denatured DNA. Hence heat-denatured *E. coli* DNA was incubated with and without IT-Dop at 55 °C for 5 min,

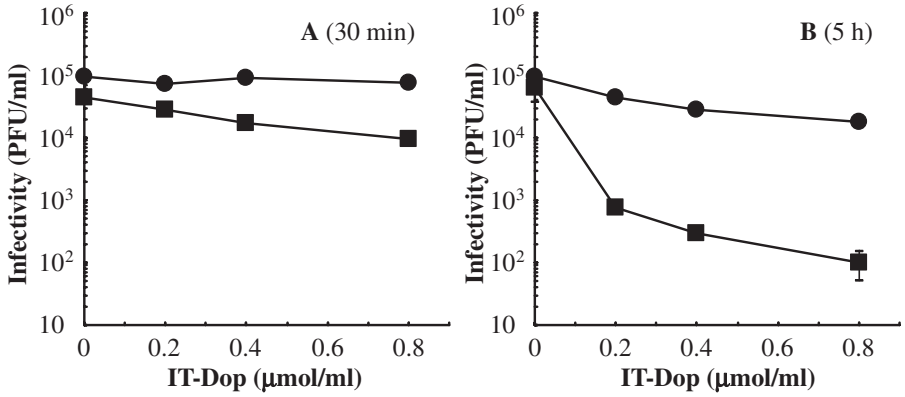


Fig. 2. The Effect of 2-(3,4-Dihydroxyphenyl)ethyl ITC on the Transfectivity of M13 Viral DNA.
M13 mp18 SS or RF DNA was mixed with the indicated concentrations of IT-Dop. After incubation at 37 °C for 30 min [A] or 5 h [B], the remaining transfectivity was determined, using Ca^{2+}-treated competent cells of *E. coli* JM109. ■, SS; ●, RF.

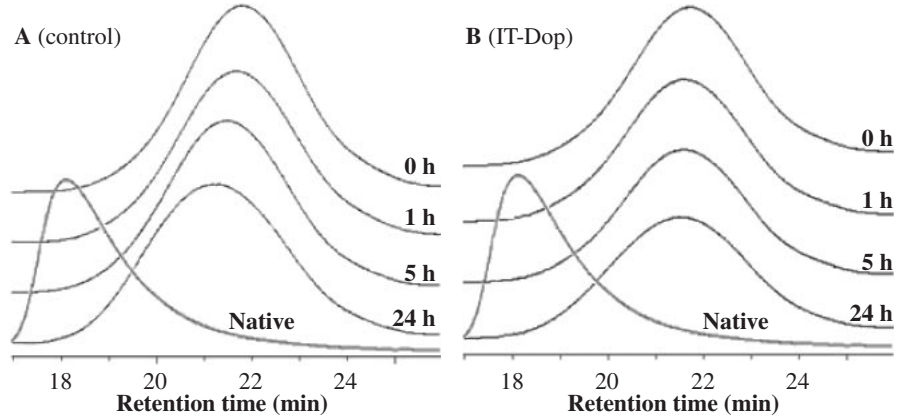


Fig. 3. GPC Chromatograms of Denatured DNA, Subjected to Annealing with or without IT-Dop.
Native *E. coli* B DNA (200 μg/ml in 50 mM Tris–HCl buffer, pH 7.6) was heated at 100 °C for 5 min and rapidly cooled in ice water. To the denatured DNA solution, IT-Dop in DMSO was added to 17 μmol/ml, and the mixture was incubated at 55 °C for 5 min, slowly cooled to 38 °C for 1 h, and then kept at 37 °C. At the indicated times, an aliquot (100 μl) was removed and subjected to gel permeation chromatography, using a TSKgel α-M column (0.78 × 30 cm). Elution was performed with 50 mM Tris–HCl buffer (pH 7.6) at a flow rate of 1.0 ml/min at 35 °C. The detection wavelength was 260 nm. A, untreated control (DMSO only); B, treated with IT-Dop.

cooled slowly to 38 °C for 1 h, and then kept at 37 °C. After incubation, an aliquot of the mixture was removed and subjected to gel permeation chromatography. As shown in Fig. 3A, prolonged incubation gradually shortened the retention time of the IT-Dop-untreated DNA, indicating the occurrence of partial renaturation of the DNA even under nonoptimal conditions. Such a shift in elution profile, as in Fig. 3A, was not observed with DNA treated with hydroxy ITC (Fig. 3B). It is probable that interaction of denatured DNA with IT-Dop prevents interstrand hydrogen bonding. As found previously, ITC reacts with proton-donor groups of biomolecules.[3] Probably, IT-Dop affects the amino groups in nucleobases of DNA, but the rate and extent of the reaction appear to be rather low.

The results presented above indicate that IT-Dop spoils the infectivity of bacterial virus ϕK and M13 single stranded DNA, whereas the double stranded RF DNA of M13 is rather refractory to the reagent. In this respect, it would be interesting to test the effect of this compound on infective phage RNA, another single-stranded polynucleotide. A free amino group of protein and DNA is probably the main target of IT-Dop, but further work is necessary to investigate any direct interaction with biopolymers, as well as to elucidate the reactive group.

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