Potassium permanganate as an *in situ* probe for B-Z and Z-Z junctions

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Received May 8, 1991; Revised and Accepted November 25, 1991

ABSTRACT

The availability of DNA structural probes that can be applied to living cells is essential for the analysis of biological functions of unusual DNA structures adopted *in vivo*. We have developed a chemical probe assay to detect and quantitate left-handed Z-DNA structures in recombinant plasmids in growing *E. coli* cells. Potassium permanganate selectively reacts with B-Z or Z-Z junction regions in supercoiled plasmids harbored in the cells. Restriction enzyme recognition sites located at these junctions are not cleaved by the corresponding endonuclease after modification with KMnO_4 . This inhibition of cleavage allows the determination of the relative amounts of B- and Z-forms of the cloned inserts inside the cell. We have successfully applied this method to monitor the extent of Z-DNA formation in *E. coli* as a function of the growth phase and mutated topoisomerase or gyrase activities. The assay can in principle be used for any unusual DNA structure that contains a restriction recognition site inside or near the structural alteration. It can be a useful tool to analyze *in vivo* correlations between DNA structure and gene regulatory events.

INTRODUCTION

Left-handed Z-DNA was proposed approximately 20 years ago (1); however, the existence of Z-DNA *in vivo* had been disputed (2) until recently, when enzymatic and topological assays were developed which demonstrated the existence of Z-DNA in living cells (3–6). An *in situ* chemical probe assay with osmium tetroxide was established, which is more sensitive than the previous assays and shows that localized supercoiling stabilizes short alternating dC-dG sequences in Z-structures in *E. coli* (7–10). In addition, potassium permanganate has been used to demonstrate DNA structural distortions *in vitro* and *in situ* (11–15). Since it has been suggested that Z-DNA could be involved in gene regulatory events (4–6, 16), the availability of a variety of sensitive and quantitative assays will be essential for the analysis of potential *in vivo* functions of left-handed DNA.

We present here a new assay for quantitating Z-DNA *in vivo* by using a combination of potassium permanganate modification and quantitation of the resulting inhibition of site-specific cleavage by restriction enzymes. Since Z-DNA formation can be used to monitor changes of supercoil densities *in vivo* (5, 17), which are influenced by environmental factors and growth phases (18–21), this assay could also be used in evaluating the relationship between these factors and supercoiling changes, as well as their influences on transcription, replication, or gene regulatory events.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used in this study were HB101, JTT1, RS2, SD7, and NH647. All strains except HB101 (hsdS20 (r-B, m-B), recA13 ara14 proA2 lacY1 galK2 rpsL20 (Smr) xyl-5 mt1-1 supE44 mcrB) are derivatives of *E. coli* K12. JTT1 (pyrF gal125 nirA strA195) is a wild type (22). RS2 (pyrF gal125 nirA strA195 top10) has a point mutation (top 10) in the gene for topoisomerase I which results in a higher level of supercoiling than that of the wild type (22). SD7 (pyrF gal125 nirA strA195 top10 gyrB266) has a top 10 mutation and a compensatory mutation in the gene for DNA gyrase which results in a lower level of supercoiling than that of JTT1 (22). NH647 (LacX74 galK2 galT22 supE44 hsdS zei 3001::TN10 gyrA43(ts)) is a gyrase A temperature-sensitive mutant which can grow only at permissive temperature (32°C), but not at 42°C (17, 23).

Plasmids

The construction and characterization of the recombinant plasmids (Fig. 1) and their capabilities to form left-handed Z-DNA *in vivo* were reported (4, 5). Plasmid pRW790 is a 1995 bp derivative of pBR322 which was constructed from pBRN3 and the polylinker region of M13mp10RF (24). Topoisomer populations with defined average superhelix densities were prepared as described (41). The average linking differences of the populations were determined by two-dimensional gel electrophoresis, with a population relaxed under KMnO_4 reaction condition as marker for zero linking differences.

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In situ permanganate reaction

Cells harboring plasmids were grown overnight in LB media plus ampicillin (100 μ g/ml). They were then diluted 1:100 from the overnight culture into the same media and grown to an OD₆₀₀ of 0.8. After centrifugation, the cells were washed twice with the phosphate buffer described above. Potassium permanganate was added to a final concentration of 5 μ M and reacted for 4 minutes at 37°C with shaking. At this time, cells were poured into chilled tubes containing 5 volumes of cold phosphate buffer and were spun for 5 min at 5000 rpm. Plasmid DNA was isolated by the alkaline lysis procedure (25) and used for subsequent fine mapping or restriction analysis.

Restriction mapping

Plasmids were digested with *Pst*I to linearize the DNA, extracted with phenol and ether and precipitated with ethanol. After the first cleavage, pRW478 was digested with *Bam*HI, whereas pRW1558 and pRW1567 were cleaved with *Eco*RI. At this point, pRW790 was added to each sample as internal control to show that the second restriction enzyme was fully active. Fragments were electrophoresed in 1% agarose gels, stained with ethidium bromide at 5 μ g/ml for 2 min, destained, and quantitated by densitometric scanning with a BioRad Model 620 video densitometer.

Fine mapping of KMnO₄ modified sites

Supercoiled plasmid substrates were isolated from *E. coli* HB101 cultures and had an average supercoil density of -0.05 . pRW478, pRW1558 or pRW1567 (1 μ g) were treated with KMnO₄ (6mM) in 30 μ l of 10mM Tris-HCl, 1mM EDTA or potassium phosphate buffer, at pH 7.5, for 2 minutes at 37°C. The reaction was then quenched by the addition of β -mercaptoethanol to 1.0 M. After ethanol precipitation, the DNA was digested with *Eco*O109 and *Eco*RV restriction enzymes. The DNA fragments were 3'-end-labelled at the *Eco*O109 sites using the Klenow fragment of *E. coli* DNA polymerase (U.S. Biochemical) and [α -³²P] dCTP for 5 min at room temperature. The labelled fragments were separated on polyacrylamide gels and the fragments of interests were eluted and purified. The DNA was then heated with 1M piperidine at 90°C for 30 minutes to cleave permanganate adducts, and analyzed by electrophoresis in 12% polyacrylamide gels containing 7M urea and 30% formamide, except for pRW1558, where formamide was omitted. Maxam-Gilbert sequencing reactions of the same fragments were run as references. For fine mapping of the KMnO₄ modified thymine residues on the linear plasmids, the DNA was first linearized using *Eco*O109 and *Eco*RV enzymes prior to KMnO₄ modification. After phenol-chloroform extraction and ethanol precipitation, the DNA was treated with KMnO₄ as described above.

Linking number analysis

Topoisomer distributions were analyzed on 0.8% agarose gels in TBE buffer containing 4mM of chloroquine (26).

RESULTS

Potassium permanganate is a pyrimidine-specific reagent that oxidizes unpaired bases in DNA (27, 28). We have tested permanganate as chemical probe for the structural distortions at B-Z or Z-Z junctions in recombinant plasmids. The concept of

Fig. 1. Plasmids used in these studies. All of the plasmids were derived from pBR322 (4, 33). The numbers of dC-dG dinucleotide repeat units are indicated underneath the brackets. The inserts are not drawn to scale. E, *Eco*RI site (G-AATTC); B, *Bam*HI site (GGATCC); X, *Xho*II site (RGATCY; R = purine, Y = pyrimidine). When the E or B sites are flanked by dC-dG dinucleotides, 4 bp are added to the total length.

B-Z as well as Z-Z junctions has been established previously using different chemical and enzymatic probes (42-44).

Three pBR322 derivative plasmids containing different lengths of alternating dC-dG inserts, were used as substrates for the permanganate reactions, whereas pBR322 served as control for an unperturbed *Eco*RI site in the vector DNA (Fig.1). Previous experiments have shown that these inserts could adopt Z-structures *in vitro* and *in vivo* (4-6).

pRW478 contains two (dC-dG)₁₃ tracts flanking a *Bam*HI site. Thus, this restriction site forms a Z-Z junction region in the supercoiled molecule (5, 24), whereas the two *Eco*RI sites at the vector-insert borders form B-Z junctions (24). pRW1558 contains an *Eco*RI site at the location of the Z-Z junction. Also, pRW1567 containing an uninterrupted 14 bp insert does not contain any Z-Z junctions, but only B-Z junction regions at the *Eco*RI and *Xho*II sites.

Permanganate modifies B-Z and Z-Z junctions *in vitro*

We performed reactions with potassium permanganate and three different Z-DNA containing plasmids (as described in Materials and Methods) and analyzed the sites of modifications at the base pair level on high-resolution sequencing gels (Fig. 2A). Plasmid pRW1567, pRW1558, and pRW478 at native superhelical densities were used as substrates; the linear forms of these plasmids served as controls. The modification pattern observed for pRW1567 clearly shows strong reactivities at both the 5' and 3' B-Z junctions. For pRW1558 and pRW478, additional strong reactivities are also observed at the *Eco*RI or *Bam*HI sites; i.e., the Z-Z junction interphase of the two out-of-phase Z-helices in each insert. For all three plasmids, no (or only very weak) modification patterns are observed for the linear forms, indicating that supercoil-stabilized Z-helices are the targets of the chemical modifications. Figs. 2B and 2C show that this modification is accompanied by an inhibition of restriction enzyme cleavage which is supercoil-dependent and parallels the supercoil-induced B to Z transition in the plasmid insert.

Permanganate as probe for Z-DNA in *E. coli* cultures

We tested the feasibility of using potassium permanganate as a Z-DNA probe in growing *E. coli* cultures. HB101 cells containing pRW1558 were grown to late log phase, and the treatment with permanganate was performed. Modification of the Z-Z junction region in the insert was detected by inhibition of restriction cleavage by *Eco*RI of the purified plasmid (Fig. 3). Lack of cleavage by *Eco*RI at the single *Eco*RI site would indicate that the site was chemically modified, i.e., that the insert had adopted

Fig. 2. *In vitro* modification of B-Z and Z-Z junctions with permanganate. A: Fine mapping of KMnO_4 modification sites in supercoiled and linear plasmids containing different lengths of dC-dG inserts. Supercoiled plasmids (designated as S) pRW478, pRW1558 and pRW1567 were treated with KMnO_4 and analyzed as described in Methods. As control experiments (designated as L), the plasmids were linearized prior to KMnO_4 treatment. The fragments containing the dC-dG inserts were analyzed for the locations of KMnO_4 modification sites on 12% polyacrylamide denaturing gels alongside Maxam-Gilbert sequencing markers. The lanes labeled A and T represent G+A and T+C, respectively. B: Supercoil-dependence of cleavage inhibition at the central *Bam*HI site in the insert of pRW478. Topoisomer populations with defined average negative superhelix densities $\bar{\sigma}$ were reacted with KMnO_4 and analyzed by *Pst*I plus *Bam*HI double digest as described in Methods. For the second restriction digest (*Bam*HI), pRW790 was added as internal control to demonstrate full activity of the enzyme. C: Quantitation of cleavage inhibition after densitometric analysis of the gel in B. The intensities of the 1185 bp band (indicative of cleavage inhibition at the central *Bam*HI site in pRW478 *) and the 784 bp band (indicative of cleavage at this site) were normalized by dividing by the size of the respective fragments. The ratio 1185 bp/784 bp of these normalized intensities (molar ratio) was plotted as a function of $\bar{\sigma}$.

a left-handed helix structure inside the cell (8–10). Since a left-handed helix by itself inhibits cleavage by *Eco*RI (29, 30), the plasmid had to be linearized with a different enzyme (*Pst*I) first, before inhibition due to permanganate reaction at the *Eco*RI site could be detected.

Fig. 3 shows that for pBR322 no modification of the *Eco*RI site had occurred in the cell, since only the larger *Eco*RI-*Pst*I and smaller *Pst*I-*Eco*RI fragments, but no full-length *Pst*I-linear molecules were found. However, for pRW1558 a substantial amount of *Pst*I-linear plasmid remained after the second digestion with *Eco*RI, indicating that the latter site was substantially modified in the cell. Concomitant with the occurrence of the *Pst*I-linear band, a decrease in the amount of the smaller *Pst*I-*Eco*RI fragment was observed, as expected.

Qualitatively similar results were observed with pRW478 and pRW1567 in HB101 cells. These data show that potassium permanganate, in combination with a suitable restriction double digestion, can be employed as an *in situ* structural probe for Z-DNA.

***In situ* analysis of KMnO_4 reactive sites in plasmids containing different (dC-dG) inserts**

To further analyze the sites of modification in the cells at the base pair level, *E. coli* HB101 harboring the plasmids with different (dC-dG) inserts were grown and treated with KMnO_4 as described in Methods. The reactivity patterns of the DNAs were compared with those obtained *in vitro*. For the DNA isolated from the cells treated with KMnO_4 , the pattern (Fig. 4) resembles the one observed with the *in vitro* modified DNAs (Fig. 2). Thus, strong reactive T's were observed on pRW478 and pRW1558 (containing 56 and 48 bp (dC-dG) inserts, respectively) in the center of the (dC-dG) stretch (Z-Z junction)

Fig. 3. Permanganate modification of Z-Z junctions *in situ*. Reactions were performed at 37°C with cell cultures at OD₆₀₀ of 0.8 and a final concentration of potassium permanganate of 5 mM. (+), DNA after permanganate modification; (–), DNA without permanganate modification. Lanes: M, 1 kb marker; a and b, pBR322 as a negative control for the permanganate reaction; c, nicked and supercoiled pRW790; d, linear pRW790; e and f, *Pst*I digested pRW1558; g and h, pRW1558 digested with *Pst*I and *Eco*RI without internal control; i and j, same as g and h, but with pRW790 added as internal control for the *Eco*RI digest.

and at both the 5' and 3'-end junctions (B-Z junction). Substantial reactivities were also observed at the junction with the vector (B-Z junction) for the 14 bp (dC-dG) insert in pRW1567. These reactive sites were also observed when the top strands were mapped (data not shown). These results indicate that the (dC-dG) tracts have undergone a B-Z transition in the cell and that KMnO_4 as a probe can positively detect such structural transitions.

Changes of *in vivo* supercoiling monitored by chemical probing of cell cultures

A combination of permanganate modification of cells, Z-DNA formation as measure of torsional strain *in vivo*, and inhibition

Fig. 4. *In situ* analyses of KMnO_4 reactive sites of plasmids containing different lengths of dC-dG inserts. Treatment of *E. coli* HB101 harboring pRW1567, pRW1558 or pRW478 with KMnO_4 , DNA isolations, and fine mapping of permanganate-modified sites were as described in Methods. The products were analyzed on 12% polyacrylamide gel containing 7M urea and 30% formamide. The sequences of the bottom strands mapped in this experiment are indicated at the right side. The arrowheads indicate the KMnO_4 reactive sites.

of restriction cleavage was used to monitor changes of the superhelical strain in plasmids during different growth stages. *E. coli* HB101 cells transformed with pRW478 were grown in LB medium for various periods of time and permanganate reactions were performed at OD_{600} values ranging from 0.2 to 1.5 (Fig. 5). The purified plasmids from each time point were analyzed by restriction double digests as described above, and the inhibition of restriction cleavage was quantitated by densitometry. In addition, plasmid topoisomer populations from parallel cultures were analyzed on agarose-chloroquine gels (Fig. 5).

At the highest cell proliferation rate (OD_{600} of 0.6), the maximum inhibition of restriction cleavage was observed (which reflects the highest amount of Z-DNA), indicating the most negative supercoiling stress at this stage. When the cell proliferation rate was close to zero at OD_{600} of 1.5, the minimum inhibition (the least amount of Z-DNA) and much less negative supercoiling were observed for the plasmid topoisomers.

Z-DNA formation in different bacterial strains

In order to test the sensitivity of this *in situ* assay, we performed the chemical modification with three plasmids (pRW1567, pRW1558, or pRW478) containing different lengths (14, 48, or 56 bp, respectively) of Z-DNA inserts, each transformed into different *E. coli* topoisomerase mutant strains (Fig. 6). JTT1 is the reference wild-type for the mutant strains RS2 (which is a top10 mutant and produces higher negative supercoiling than JTT1) and SD7 (which is a gyrase mutant and produces less negative than wild-type supercoiling levels) (22; Materials and Methods). The percentages of inhibition of restriction cleavage found in each strain, as detected by the extent of chemical modification at the *Eco*RI or *Bam*HI sites, were quantitated as

Fig. 5. Correlation between plasmid linking number distribution, amount of Z-DNA, and cell proliferation. Permanganate modifications were performed with *E. coli* HB101 harboring pRW478 for four minutes at 37°C with a permanganate concentration of 5 mM. A: At different OD_{600} points, plasmid DNA was isolated from cells grown in parallel, but without permanganate modification, and the topoisomer distributions were analyzed on a 0.8% agarose gel containing 4 μM chloroquine (migration was from top to bottom). B: At different OD_{600} points (open squares), the amounts of Z-DNA (in percent of total plasmid DNA, filled squares) were determined after permanganate modification as described in Materials and Methods.

described above. Fig. 6 shows that in all four strains, larger inserts formed Z-DNA to a higher extent than shorter inserts, as observed previously (5, 26). By using our new assay even the shortest insert (14bp) was modified by permanganate to a measurable extent (4, 5). Similar modifications *in situ* of very short dC-dG inserts were observed recently using osmium tetroxide as chemical probe (7).

In addition, Fig. 6 shows that in HB101 and JTT1 the extents of modification are quite similar for all three lengths of inserts, and that in SD7 the amounts of Z-DNA were lowest for all three plasmids, as expected (17). RS2 gave the highest signal for Z-DNA formation in the 14bp insert (pRW1567). Surprisingly, however, the signals for the 48bp and 56bp inserts were not much different or even slightly lower in this strain compared to HB101 and JTT1. An interpretation of this unexpected behavior is discussed below.

DISCUSSION

A new assay is described for the detection of left-handed Z-DNA in recombinant plasmids *in vitro* and *in situ*. Millimolar amounts of potassium permanganate modify distorted helix segments at B-Z and Z-Z junction regions, as determined by mapping of the sites of modification at the base pair level. The main targets are T-residues and to a minor extent also C- and A-residues at these junctions. Inhibition of cleavage at restriction sites located at these junctions, caused by the chemical modification of bases, is used to quantitate the relative amounts of Z-DNA in a population of plasmid molecules.

The selectivity of permanganate for distorted regions of the DNA helix, without modifying the unperturbed B-helix structure, is demonstrated by the lack of cleavage inhibition at other restriction sites (*Pst*I, *Sal*I, *Hinc*II, *Ava*I) further away from the Z-DNA region (data not shown) or at restriction sites in pBR322

Fig. 6. Quantitation of Z-DNA formation in topoisomerase mutants. Each of the three plasmids with different lengths of inserts, transformed in four different *E. coli* strains, were analyzed after permanganate modifications *in situ*. The amounts of Z-DNA formed inside the cells were measured as described and are shown as percentages of total plasmid DNA. For each case, the average of 3 individual experiments is shown; the reproducibility was within 10% or less.

without any insert. It is clear especially for pRW478 with two *Bam*HI sites that the *Bam*HI site inside the Z-DNA insert is substantially modified, whereas the *Bam*HI site 400 base pairs away from the insert does not react with permanganate under controlled conditions.

When analyzing recombinant plasmids in growing *E. coli* cultures, treatment with 3 to 5 mM permanganate destroys the ampicillin resistance in more than 95% of the cells (as determined by spreading of reacted cell cultures on plates with or without ampicillin (data not shown). Thus, we consider this procedure to be an *in situ* and not a true *in vivo* assay. However, based on the results discussed below obtained with different lengths of Z-inserts and different *E. coli* strains, we believe that during the short reaction time (a few minutes) the intracellular milieu of the cell is largely unaltered by the chemical modification step.

The reactivity and selectivity of potassium permanganate are quite similar to osmium tetroxide, which has been used as a DNA structural probe for B-Z junctions *in vitro* and *in situ* (7–10). However, permanganate at millimolar concentrations is a convenient chemical without the need for special precautions during handling, whereas great care must be taken during the use and disposal of osmium tetroxide solutions. More important, the combination of permanganate modification *in situ* and inhibition of restriction cleavage allows a reliable quantitation of the relative amounts of B- and Z-forms in a plasmid population, whereas quantitative determinations have not been developed for osmium tetroxide modifications. As discussed above, permanganate possesses a high selectivity under the conditions used here. Since the redox potential of permanganate is strongly dependent on the pH of the reaction medium, it should be possible to vary or further increase this selectivity by pH variations, in order to adapt to different reactivities at distorted DNA regions in other types of unusual DNA structures or other host cells.

We performed a series of *in situ* modification experiments to show that permanganate can monitor changes in the relative amounts of Z-DNA under different intracellular conditions in a reliable and reproducible way. Correlations between *in vivo*

plasmid supercoiling levels, and thus stability of supercoil-induced Z-structures, and the growth phases of *E. coli* cultures have been analyzed (17–21). Our results show that potassium permanganate can detect the changes in the extent of Z-DNA formation in the 56 bp insert of pRW478 which result from growth phase-induced variations of the *in vivo* supercoiling levels of the plasmid.

It was shown previously with less sensitive assays that longer dC-dG inserts form Z-DNA in *E. coli* to a higher extent than shorter inserts (5, 17, 26). We observed the same length-dependent behavior using permanganate as a probe. More important, permanganate was able to detect even minor amounts of Z-DNA (approximately 10%) in a 14 bp insert which did not produce any Z-DNA signal with enzymatic or topological *in vivo* assays (5, 17). Thus, permanganate is as sensitive as the osmium tetroxide *in situ* assay described previously (7).

The activities of topoisomerase I and DNA gyrase have an impact on the *in vivo* supercoiling levels in *E. coli*, thus effecting the stabilities of the Z-form in dC-dG inserts of varying lengths (17, 22). Our experiments with the topoisomerase mutant strains RS2 (top10 mutant) and SD7 (gyrase mutant) as well as the wild-type strains HB101 and JTT1 clearly show that inactivation of the DNA supercoiling activity of gyrase in SD7 decreases the amounts of Z-DNA in all three lengths of inserts studied. Inactivation of the relaxing activity of topoisomerase I in RS2 doubles the small amount of Z-DNA observed in the other strains for the 14 bp insert. although RS2 as a topoisomerase I mutant possesses higher levels of *in vivo* supercoiling than HB101 and JTT1 (17, 22) surprisingly, the two longer inserts of 48 bp and 56 bp in this strain behaved quite similarly as in the wild-type strains HB101 and JTT1. The reason for this observation may be similar to what we have observed with our *in vitro* experiments which also did not yield 100% modification for the populations that definitely had 100% Z-DNA. Alternatively, it may be caused by a supercoiling-dependent inactivation of the tetracycline promoter in conjunction with transcription-induced effects on localized plasmid supercoiling (6, 7). A higher than wild-type negative supercoiling level in RS2 was observed using a topological linking number assay (17). This assay measures the global supercoiling of the plasmids, whereas the permanganate assay measures Z-DNA formation due to the local supercoiling superimposed on the global supercoiling at the location of the insert. Since the dC-dG blocks are located upstream of the tetracycline promoter, a possible inhibition of promoter activity by a higher global superhelical density might indeed lead to a lower than expected local supercoiling at this site, and thus to a lower than expected amount of Z-DNA formation. The observation that pBR322, transformed into RS2, does not render this strain resistant to tetracycline supports this interpretation (17).

Potassium permanganate has been used recently for the *in vitro* analysis of triple-helical DNA isomers (31), a supercoil-promoted bent loop region in *lac* DNA (32), and protein contact sites in a hormone-responsive regulatory element (14), as well as for *in situ* analyses of protein-induced DNA structural alterations in the SV40 ori and major late promoter regions (12, 13, 33) or in the *lac* and *glnALG* control regions of *E. coli* (11, 28). These studies, in conjunction with the new quantitative approach presented here, demonstrate the wide potential of potassium permanganate as a tool for the analysis of gene regulatory elements.

Previous studies show that changes of supercoiling can occur as a response to environmental stimuli such as nutrition, osmotic shock, anaerobiosis etc. (18–21, 34–36). Supercoil stabilized non B-DNA structures have been shown to influence transcription

and replication processes *in vitro* (37–40). The extent of these non B-DNA structures have not been determined. Since the required reaction time of KMnO_4 is faster than the other known probes, it could be used as a probe for detection and quantitation of relative amounts of DNA structural features *in situ*.

ACKNOWLEDGMENTS

We thank Dr. Robert D. Wells for his interest and support of these studies. This work was supported by a Biomedical Research Award from the Arthritis Foundation and grants GM30822 from the National Institutes of Health, 8607785 from the National Science Foundation. Preliminary experiments were performed in collaboration with Drs. K. Negishi and H. Hayatsu, Department of Pharmaceutical Chemistry, Okayama University, Japan, which were supported by a travel grant from the Japan Society for the Promotion of Science and the American Cancer Society.

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