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Adenosine–guanosine preferential photocleavage of DNA by azido-benzoyl- and diazocyclopentadienylcarbonyloxy derivatives of 9-aminoacridine

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ABSTRACT

The photoreactions of 9-[6-(4-azidobenzamido)hexylamino]acridine (AHA) and 9-[6-(2-diazocyclopentadienylcarbonyloxy)hexylamino]acridine (DHA) with double stranded DNA result in formation of single strand nicks and alkali labile sites (adducts) with an efficiency of $6x10^{-3}$ nicks per AHA and $3x10^{-2}$ nicks per DHA molecule. The alkali dependent DNA cleavage by AHA shows a pronounced A+G preference whereas that by DHA is practically sequence independent. In the presence of diacridines, however, DHA exhibits a preference for cleavage at guanosines. These DNA photocleaving reagents could be useful for DNA photofootprinting and photosequencing.

INTRODUCTION

Synthetic reagents which cleave DNA are of considerable interest as tools in molecular biology. This has led to the development of both sequence specific DNA cleavers[1-3] and DNA footprinting reagents.[4-8] In most of these cases the cleavage was carried out by transition metal complexes or by organic dyes. Redox reactions of the metal complexes produce radicals which can attack the deoxyribose units of the DNA backbone thereby leading to strand scission.[1,2,4-9] The organic dyes can oxidize the DNA bases (preferentially guanosines) *via* singlet oxygen or *via* electron transfer to give single strand breaks or alkali labile sites.[3,10-12]

Furthermore, it was recently shown that oligonucleotide linked azidophenacyl[13] or azidoproflavine[14] ligands and azido substituted 9-aminoacridines,[15] photoreact efficiently with DNA to give alkali labile adducts.

We now report that 4-azidobenzamido and diazocyclopentadienyl derivatives of 9-aminoacridine (Figure 1), which were previously shown to react efficiently with DNA,[16] also induce photocleavage of DNA. The azidobenzamido derivatives show a preference for pu-

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rine sites indicating that they function *via* base alkylation whereas the diazo derivative, which show very little sequence dependent DNA cleavage, probably reacts with the deoxyribose units. For the latter reagent a pronounced G preference can be provoked by adding diacridines.

MATERIALS AND METHODS

Chemistry.

Reagents 1b (AHA), 2 (DHA)[16] and N,N'-di-9-acridinylspermidine (5)[17] were prepared as previously described. Compounds 1a, 1c, 2a and 2b (Figure 1) were prepared using the procedure previously described[16] and their structures were confirmed by spectroscopic (^{1}H NMR, IR) and analytical (C,H,N) analyses.

Plasmid relaxation assay.

pUC 19 DNA was transformed into E. coli JM 101 and isolated by the alkaline extraction procedure.[18] In a typical experiment 0.3 µg DNA in 10 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was mixed with 1 µl of the reagent dissolved in TE (diluted from a stock solution of 10 mg/ml in dimethyl sulfoxide), and irradiated in Eppendorf tubes from above at room temperature using a Philips TL 40W/03 ($\lambda \sim$ 420 nm, 20 J x m^{-2} x sec^{-1}), or a Philips TL 20W/12 ($\lambda \sim$ 300 nm, 22 J x m^{-2} x sec^{-1}) fluorescent light tube at a distance of 15 cm. The DNA was analyzed by electrophoresis in 1% agarose in 0.5 x TBE buffer (45 mM Tris-borate, 0.5 mM EDTA, pH 8.3).

Photocleavage of ^{32}P-end labeled DNA fragments.

The 90 and 232 bp EcoRI/Pvu II fragments of the plasmid pUC 19 were used to study DNA cleavage at the nucleotide level. The fragments were 3'-end-labeled at the EcoRI site and purified using standard techniques. In a typical cleavage reaction 0.1-0.2 pmol of end-labeled fragment was mixed with 0.25 µg (~ 380 pmol base pairs) calf thymus DNA in 100 µl TE. After addition of 5 µl reagent (diluted as specified with H_2O from a stock in dimethyl sulfoxide (10 mg/ml)) the samples were irradiated from above with 365 nm light (Philips TL 20W/09, 22 J x m^{-2} x s^{-1}) for 30 min. This lamp was chosen as a compromise since we have found that pyrimidine/pyrimidone adducts, which give strand scission upon piperidine treatment, are produced by the TL

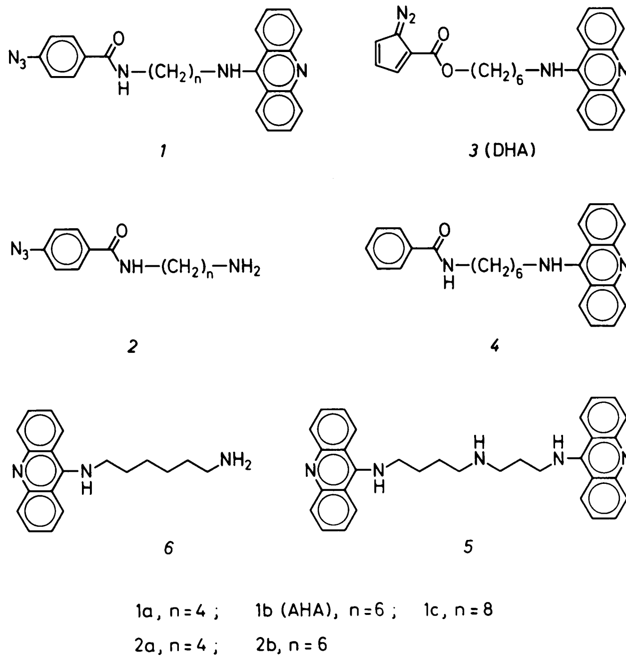


Figure 1. Structure of the reagents.

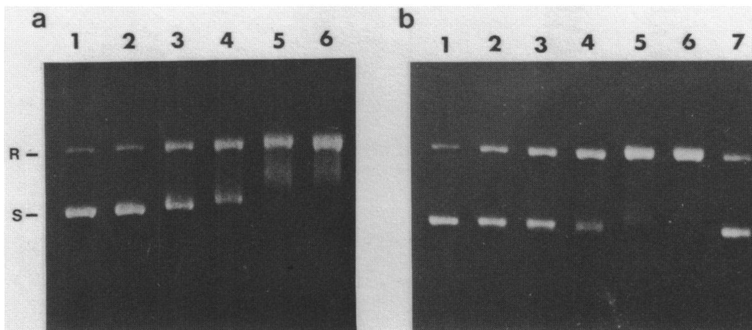
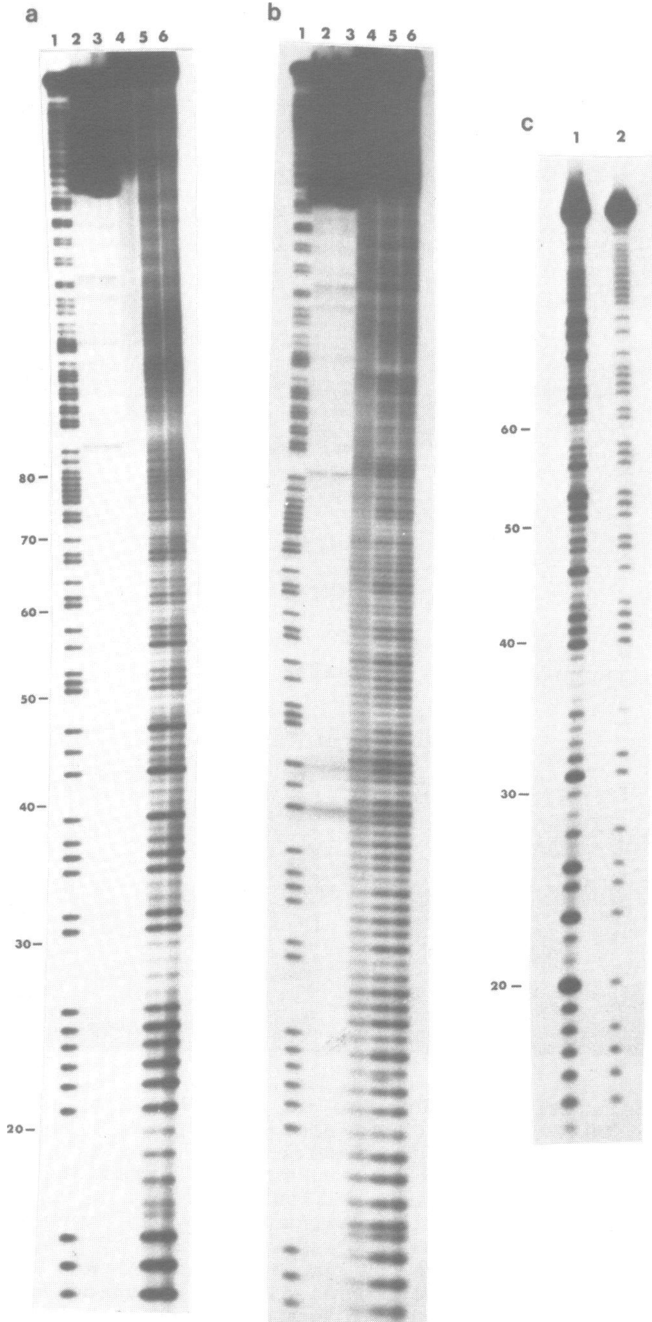


Figure 2. Relaxation of supercoiled pUC 19 DNA by photoreaction with AHA or DHA. 0.3 µg (0.45 nmol base pairs) pUC 19 DNA was mixed in 10 µl TE with 50 ng (0.1 nmol) of AHA (a) or 10 ng (0.02 nmol) of DHA (b). The samples were irradiated at 300 nm (24 J x m^{-2} x sec^{-1}) for the following times: a) Lanes 2-6: 0, 1, 2, 5 & 10 min (lane 1: no reagent), or b) lanes 1-7: 0, 5, 10, 30, 60, 120 & 0 sec. The DNA was analyzed by gel electrophoresis in 1% agarose (0.5 x TBE buffer). The gel was stained with ethidium bromide and photographed. S: super-coiled circular plasmid, R: relaxed circular plasmid. Similar results were obtained in 10 mM Na-phosphate or Na-acetate buffer (pH 7).


a
1 2 3 4 5 6
80 —
70 —
60 —
50 —
40 —
30 —
20 —
b
1 2 3 4 5 6
c
1 2
60 —
50 —
40 —
30 —
20 —

d

```
                   20                  40                  60
          .         .         .         .         .         .
3'AGCTCGAGCCATGGGCCCCTAGGAGATCTCAGCTGGACGTCCGTACGTTCGAACCGCATT

                   80                 100                 120
          .         .         .         .         .         .
  AGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGTGTGTTGTAT

                  140                 160                 180
          .         .         .         .         .         .
  GCTCGGCCTTCGTATTTCACATTTCGGACCCCACGGATTACTCACTCGATTGAGTGTAAT

                  200                 220
          .         .         .         .         .
  TAACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGCACAGCACGGTC


                   20                  40                  60
          .         .         .         .         .         .
3'AGATGACCGGCAGCAAAATGTTGCAGCACTGACCCTTTTGGGACCGCAATGGGTTGAATT

                   80
          .         .         .
  AGCGGAACGTCGTGTAGGGGGAAAGCGGTC
```

Figure 3. Photocleavage of the 3'-^{32}P-labeled EcoRI-PvuII pUC 19 DNA fragments by AHA, DHA & 2a. All samples contained 0.1-0.2 pmol of ^{32}P-endlabeled fragment and 0.25 µg (~0.36 nmol) CT-DNA in 100 µl TE and irradiations were performed at 365 nm (30 min). a,b: lane 1: A+G formic acid sequence reaction, lane 2: no reagent + irradiation, lane 3: no irradiation + reagent (a: 0.5 µg (~ 1 nmol) AHA, b: 1 µg (~ 2 nmol) DHA), lanes 4,5: reagent (a: 0.25 µg AHA, b: 0.5 µg DHA) + light (all samples except that of lane 4 were treated with piperidine prior to gel analysis), lane 6: reagent (a: 0.5 µg AHA, b: 1 µg DHA + light. c: lane 1: 1 µg 2a + light; lane 2: A+G sequence reaction. d: DNA sequences of the fragments used in a & b: 232 bp, c & Fig. 4: 90 bp pUC 19 EcoRI-PvuII.

20W/12 (300 nm) lamp (Nielsen, Jeppesen, Egholm and Buchardt, submitted). The DNA was recovered by EtOH precipitation and treated with 1 M piperidine at 90°C for 20 min followed by precipitation with 10 vols of 1-butanol. The DNA pellet was washed with 70% EtOH and lyophilized. The samples were subsequently analyzed on 8% PAG/50% urea sequencing gels and the cleavage products visualized by autoradiography.

RESULTS

The 4-azidobenzamidohexylamino- and 2-diazocyclopentadienylcarbonyloxyhexylaminoacridines, (AHA and DHA, Figure 1) photoreact very efficiently with DNA,[16] and as shown in Figure 2 the photoreaction also results in single strand scissions, measured

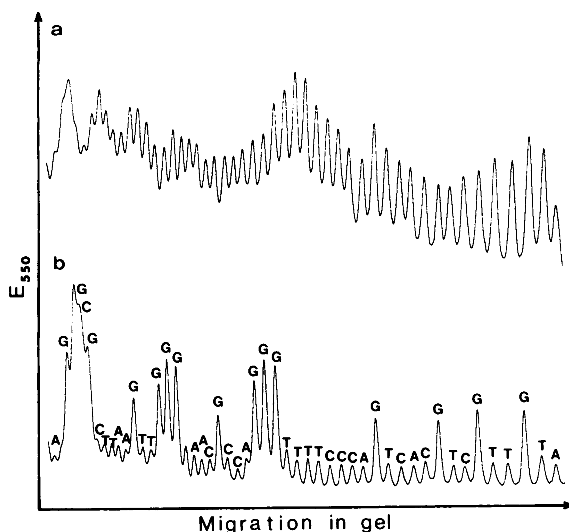


Figure 4. Sequence preference of the photocleavage of the 90 bp EcoRI-Pvu II fragment of pUC 19 by DHA (10 μg/ml) (a). b) Ditto in the presence of 10 μg/ml of N,N'-bis(9-acridinyl)spermidine.[17] The densitometric scans of the autoradiograms are shown.

by a plasmid relaxation assay. It is furthermore observed that the migration of the non-cleaved supercoiled DNA is progressively retarded as a function of photochemical formation of covalent adducts which do not result in spontaneous cleavage but which, due to the intercalating 9-aminoacridine ligands, induce DNA unwinding (this is particularly clear for AHA).

Cleavage at a considerable number (all?) of the adducts is induced by subsequent treatment with piperidine (Figure 3). These results also show that DNA photomodification by DHA occurs with only little base specificity showing least cleavage at adenine residues, whereas photomodification by AHA is highly purine (A and G) specific.

In the presence of DNA-saturating concentrations of the diacridine 5 (Figure 1), DHA exhibits a pronounced preference for modification at guanosines due to suppresion of other modification sites (Figure 4).

The number of nicks induced per reagent molecule (at the concentrations giving 50% relaxation of pUC 19) was calculated (Table I) in order to obtain a measure of the absolute DNA

Table I. Photonicking of Plasmid DNA. Relative Efficiences.

Reagent	μM+	Relative Efficiency	r+	Yield*
1a	6	30	0.13	2×10^{-3}
1b (AHA)	2	100	0.04	6.3×10^{-3}
1c	2	100	0.04	6.3×10^{-3}
2a	20	10	0.4	6.3×10^{-4}
2b	40	5	0.8	3.1×10^{-4}
3 (DHA)	0.5	400	0.01	2.5×10^{-2}
4	>200	<1		
6	>200	<1		

+The concentration and reagent/base pair ratio (r) giving 50% relaxation of supercoiled pUC 19 DNA is reported (cf. Figure 5). A light dose of 0.4 J/cm^{-2} (30 min) at 300 nm was used in all experiments. DNA concentration = 0.03 μg/μl 45 μM.

*Yield = number of single strand nicks induced per reagent molecule at the reagent concentration causing 50% relaxation corresponding to 0.68 nicks/plasmid (Poisson distribution). Assuming that all reagent molecules are bound to DNA the yield = $\frac{0.68}{r \times 2686}$ (pUC 19 contains 2686 base pairs).

photonicking efficiency of the reagents. These values are expected to be only slightly higher than the quantum yields for photonicking, based on the high quantum yields for photolysis of aromatic azides[23] and diazo compounds.

From the results presented in Fig. 3a,b (lanes 4 and 5) it can be estimated by densitometric scanning that treatment with piperidine causes a 2-3 fold increase in the number of DNA nicks produced by photoreaction of DHA with DNA, whereas the increase in the case of AHA cannot be accurately assessed (> 10 fold).

The photonicking efficiency of the azidobenzamido reagents is only slightly influenced by changes in the length (n) of the polymethylene linker ($4 < 6 \sim 8$) connecting the photoprobe with the DNA intercalating 9-aminoacridine ligand. However, the latter is important for the efficiency (Figure 5, Table 1, compare AHA (1b) with 2b).

The low photonicking activity of reagents 2a,b which lack the

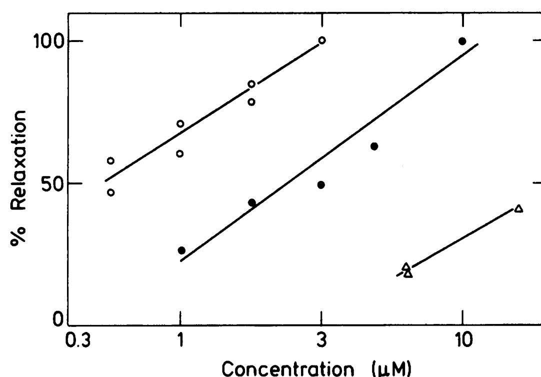


Figure 5. Concentration dependence of the photocleavage reactions. From experiments analogous to the one described in Figure 2 the influence of reagent concentration on the photocleavage was measured by varying the reagent concentration and keeping the irradiation fixed (300 nm, 10 min). The quantitation of supercoiled versus relaxed plasmid was performed by densitometric scanning of the photographed gels. DHA: o-o-o. AHA: ●-●-●, 2a: Δ-Δ-Δ. The "background relaxation" originating from the plasmid preparation has been subtracted.

DNA intercalating 9-aminoacridinyl group is probably caused by low affinity for the DNA. However, these simple reagents also react preferentially with the purine bases of the DNA (Figure 3c), which strongly indicates that selective photochemistry of the azidobenzoyl ligand and not intercalation by the acridine is responsible for the purine preference.

The wavelength dependency of the photonicking by DHA (Figure 6) is as expected from the absorption spectrum.[16] The reaction is far more efficient using 300 nm light, corresponding to absorption by the diazocyclopentadienyl ligand, compared to 420 nm, where the acridinyl chromophore absorbs. In contrast, photonicking by AHA is efficient at 420 nm analogously to its photobinding to DNA.[16,17] Thus photonicking due to the azidobenzoyl group can occur efficiently with light absorbed by the acridinyl moiety. This is probably due to an intramolecular electron transfer mechanism (Shields, Falvey, Schuster, Buchardt & Nielsen, in preparation) between the acridinyl ligand and the azidobenzoyl ligand. The photonicking of pUC 19 DNA by AHA and DHA is somewhat inhibited by Mg^{2+} or Na^{+} (Table II) but is not

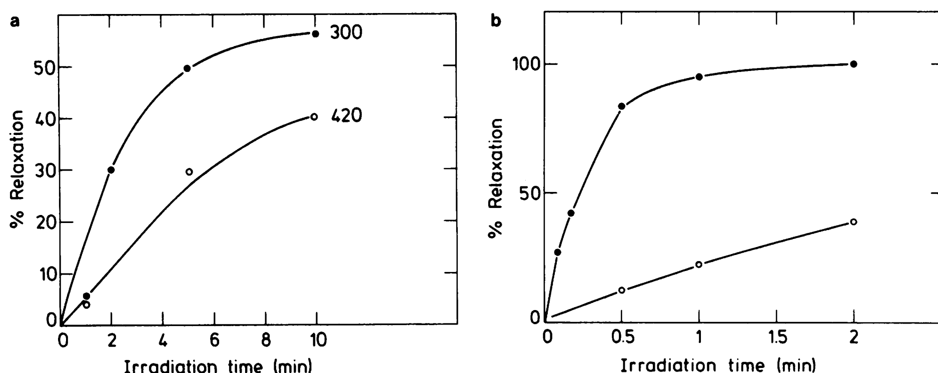


Figure 6. Irradiation and wavelength dependence of the photocleavage reactions. In an experiment analogous to the one described in Figure 2 (50 ng of AHA (a) or 10 ng of DHA (b), respectively) the samples were irradiated at either 300 nm (●-●-●) or 420 nm (o-o-o) and the photocleavage was quantitized by densitometric scanning.

affected by N_3^- (10 mM) or glycerol (5%) (data not shown). Dithiothreitol (10 mM) causes 70% inhibition of the photonicking by AHA, whereas it has no effect when DHA is used (Table II).

The inhibition of the photonicking by sodium or magnesium ions could be due to a decrease in the DNA affinity of the acridines in the presence of cations. The lack of effect observed with added azide ion or glycerol strongly argues against the in-

Table II. Photonicking of pUC 19 DNA by AHA & DHA.

Conditions	Relative photocleavage reagent 1b (AHA)	reagent 3 (DHA)
TE	100	100
+ 1 mM $MgCl_2$	44	100
+ 5 mM $MgCl_2$	-	70
+ 10 mM $MgCl_2$	20	-
+ 20 mM NaCl	87	93
+ 50 mM NaCl	62	70
+100 mM NaCl	48	66
+ 10 mM DTT	31	100

volvement of singlet oxygen or long-lived diffusing radicals in the DNA photonicking by AHA and DHA. The dithiothreitol quenching of the DNA photonicking by AHA is consistent with photoreaction via a cycloazaheptatetraene intermediate generated from the azidophenyl group,[20,21] which reacts very efficiently with thiols.[22]

DISCUSSION

The almost total lack of base specificity observed with DHA compared to the pronounced A+G specificity observed with AHA strongly indicates two different mechanisms for their action.

For AHA we suggest that the DNA cleaving is caused by light-induced attack almost exclusively at the purine bases. These are more susceptible to electrophilic attack of the photogenerated reactive species from the azidoaryl group, *i.e.* an azacycloheptatetraene.[20] This leads to some extent to spontaneous depurination and subsequent spontaneous β-elimination to form the 5'-phosphates. The piperidine treatment serves to remove all, or most of the rest of the modified purines, again followed by β-elimination.

The lack of base preference obtained with DHA is in good correspondence with its photochemical formation of a carbene species, which inserts non-discriminately. Subsequent DNA cleaving, spontaneous or piperidine induced should lead to lack of base specificity. This result also shows that DHA binding to DNA is neither base nor sequence specific, which is presumably common for other related 9-aminoacridines. The β-elimination, which would lead to 5'-phosphates is evidenced by the observation that radiolabeled DNA fragments were formed, which co-migrate with the 5'-phosphates generated in the Maxam-Gilbert sequencing reactions.

The geometry of the intercalating 9-aminoacridine ligand is believed to position the linker in the minor groove,[24] and thus determine that the attack of the photoprobes likewise takes place in the minor groove.

The G-specificity provoked by diacridine (or a triacridine but not 9-aminoacridine itself, data not shown) on the DHA induced cleavage is not fully understood. However, a similar phe-

nomenon was observed with DNase I digestion of DNA in the presence of oligoacridines,[19] and we suggest that it reflects the conformation of the DNA-oligoacridine complex rather than being a simple footprint of the acridine binding site on the DNA.

The base specific photocleavage of DNA by AHA can be used as part of a photochemical DNA sequencing strategy, for which T[24] and G[3,10] specific photocleavage reactions already exist. In principle all the information necessary for DNA sequencing can be obtained by photochemical methods if the sequence independent base ladder produced by DHA is included. By analogy to the use of EDTA linked to DNA binding drugs,[1] oligonucleotides,[1,26] or peptides,[27] azidobenzoyl or diazocyclopentadienyl ligands may be used in photochemically activated sequence directed DNA modifying reagents. Finally, AHA and DHA are also potential photofootprinting reagents that may be used in studies of protein-(ligand)-DNA interactions. These aspects are now being examined.

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