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Biological Evaluation of Dicyanoisophorone based Azo Compound: Antimicrobial and Antioxidant Properties

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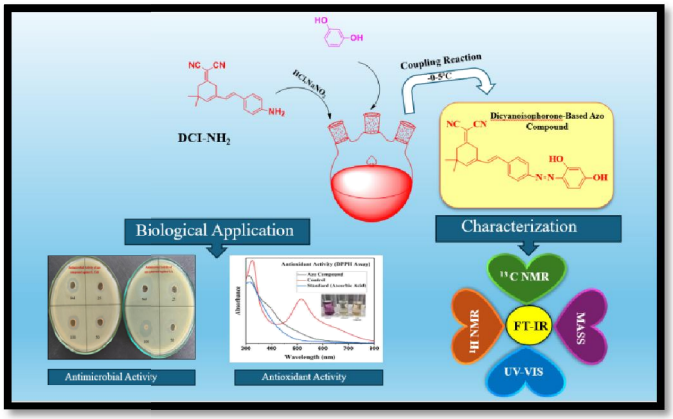
Abstract: *In continuation of our earlier report on the optical and sensing properties of a dicyanoisophorone-based conjugated azo compound and its disclosure in a published patent, the present study explores the biological potential of the same molecular framework. The compound was synthesized using a conventional diazotization-coupling strategy and characterized by FT-IR, UV-Visible, and NMR spectroscopy. The biological activity of the compound was evaluated through in-vitro antimicrobial and antioxidant assays. The synthesized molecule exhibited appreciable antibacterial activity against selected Gram-positive and Gram-negative bacterial strains. Additionally, the compound demonstrated significant free-radical scavenging ability in the DPPH assay, indicating its antioxidant potential. The results suggest that the previously reported optically active molecule also possesses promising biological properties, thereby extending its application scope beyond optical and sensing domains.*

Highlights:

- *A dicyanoisophorone-based azo compound previously reported for optical properties was biologically evaluated.*
- *The same molecular framework disclosed in a published patent was explored for antimicrobial activity.*
- *The compound exhibited effective inhibition against both Gram-positive and Gram-negative bacteria.*
- *Significant antioxidant activity was observed through the DPPH radical scavenging assay.*
- *The study demonstrates the multifunctional potential of a single molecular system beyond optical applications*

Graphical Abstract: Biological applications of Dicyanoisophorone based Azo Compound



Keywords: Dicyanoisophorone based azo compound; Antimicrobial Activity; Antioxidant Activity

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I. INTRODUCTION

The cyano group(–C≡N) has been extensively studied for its role in enhancing biological activity by improving interactions with enzymes**,** receptors**,** and microbial targets. Due to its strong electron-withdrawing nature and small size, the cyano group can significantly influence binding affinity**,** membrane permeability, and overall bioavailability[1]. In various biological assays, cyano-containing compounds have demonstrated notable antibacterial**,** antifungal**,** and anticancer properties[2].

Azo compounds are significantly important in biological studies due to their broad pharmacological activities. These compounds have demonstrated antibacterial, antifungal, anti-inflammatory, and anticancer properties. The biological activity of azo derivatives is influenced by the nature and position of substituent groups on their aromatic rings, which affect their interaction with biological targets such as enzymes, DNA, and cell membranes. In addition, azo compounds are utilized in prodrug strategies, where the azo bond is enzymatically cleaved in the body to release active therapeutic agents, especially in targeted colon-specific drug delivery systems. Their structural versatility and bioactivity make azo compounds important candidates for ongoing research in medicinal and pharmaceutical sciences[3].

Dicyanoisophorone based molecular frameworks are emerging as potent tools in biological imaging and sensing, owing to their strong intramolecular charge transfer (ICT) and near-infrared (NIR) fluorescence properties, which enable deep-tissue visualization with minimal background interference[4]. In our earlier work, we reported the optical, solvatochromic, and sensing properties of a dicyanoisophorone-based conjugated azo compound, demonstrating its excellent photophysical performance. The same molecular entity has also been disclosed in a published patent, highlighting its structural novelty and functional relevance. However, despite these advances, the biological potential of this molecular framework has not been systematically explored [5].

Recent studies have shown that dicyanoisophorone-based compounds exhibit promising biological applications, including the selective detection of biologically relevant species such as cysteine (Cys), selenocysteine (Sec), glutathione (GSH), and acetylcholinesterase (AChE), with advantages such as high photostability, low cytotoxicity, and efficient cellular uptake [6]. These attributes make such compounds valuable for real-time imaging, tumor diagnosis, and redox monitoring in live biological systems.

Furthermore, Dicyano-substituted azo compounds, along with those incorporating isophorone or thiadiazole scaffolds, have garnered increasing attention as promising candidates due to their combined antimicrobial and antioxidant properties[7].Their biological activity is mediated through several actions, including breaking down microbial membranes, counteracting reactive oxygen species (ROS), and suppressing critical enzymes that regulate cellular functions[8]. As noted by Benkhaya et al., these compounds hold significant potential for Use in chemical sensing and medical technologies, underscoring the relevance of their classification, physicochemical properties, and contemporary synthetic strategies [9].

From the literature survey, it was confirmed that the molecular framework containing Cyano and/azo group are important in medicinal chemistry. In this regard, we envisioned the molecular structure containing both cyano and azo group in single nucleous which can integrate the biological properties of the both functionality in same molecule. Here, in this work, we report the synthesis and structural characterization of a newly designed azo compound based on dicyanoisophorone and resorcinol, with a focus on its biological activities, particularly its antimicrobial and antioxidant effects. Through a combination of spectroscopic techniques (^{1}H-NMR, ^{13}C-NMR, FTIR, mass spectrometry, and UV-Vis), the compound's structure was confirmed. Its biological potential was assessed through in vitro assays, including the agar well diffusion method against E. coli and S. aureus, as well as the DPPH radical scavenging assay. The study aims to contribute to the development of multifunctional azo compounds with promising biomedical applications.

II. MATERIAL AND METHODS

2.1 Pathogenic strain: Antibacterial activity of the synthesized dicyanoisophorone based azo compound with Standard reference strains of pathogens *Staphylococcus aureus* gram-positive (23235) and *Escherichia coli* gram-negative (25922). *E. coli* (25922) and *S. aureus*(23235) are the ATCC numbers of microorganisms. All the test organisms were

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obtained from the Cell Culture Laboratory, Department of Biotechnology, M.P.A.S.C. College, Panvel, Raigad, India. (University of Mumbai)

2.2 Culture media and reference antibiotics: Sterile Mueller-Hinton agar is used to inoculate all bacterial isolates.

2.3 Chemistry:

Chemicals from Sigma-Aldrich and Merck were used without further purification. NMR spectra were recorded on a 600 MHz Bruker spectrometer, and HR-MS data were obtained using UHR-TOF (EI mode). IR, UV-Vis, and fluorescence spectra were measured using Shimadzu 8400, UV-1800, and RF-6000 instruments, respectively. Melting points were determined electro thermally without correction. Dicyanoisophorone based azo Compound purity was confirmed by HPLC (Alliance Waters 2697).

2.4 Experimental:

The method given below by Yu Cheng et al. was used to synthesise compounds 1, 2, and 3 [10][11].

Synthesis of Dicyanoisophorone based azocompound:

Diazotization was carried out at 5 °C using sodium nitrite ($NaNO_2$, 1 mmol) on a solution of Compound 3 (0.198 g, 1 mmol) in acidified water. The resulting diazonium salt was subsequently coupled with resorcinol (0.22 g, 2 mmol) in ethanol, followed by the gradual addition of 10% NaOH, while maintaining the reaction temperature between 0 and 5 °C. The appearance of a red coloration confirmed successful coupling. The reaction mixture was allowed to stand for 60 minutes, after which the pH was adjusted to 6 and the solid was left to settle for 24 hours[12][13]. Recrystallisation from dichloromethane yielded the azo compound with 84% recovery and a melting point of 110 °C. Purity was confirmed by HPLC using an acetonitrile: water (9:1) solvent system.

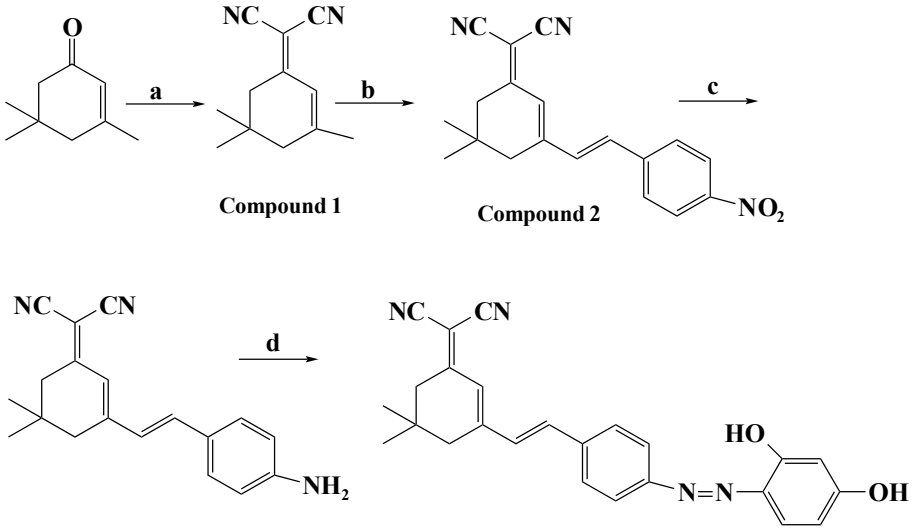

Figure.1. General Scheme for the synthesis of the Dicyanoisophorone based azo Compound.

Reactant and conditions: (a) Malanonitrile, CH_3COOH, 6h, 84%; (b) piperidine, 4-nitrobenzaldehyde, 6h, 76%; (c) HCl, $SnCl_2 \cdot 2H_2O$, 8 h, 70%; (d) $NaNO_2$, HCl, EtOH, Resorcinol in NaOH, Cooling 0-5˚C,84%.

2.5 Antimicrobial Activity of Synthesized Dicyanoisophorone based azo Compound:

The agar well diffusion method was employed to evaluate the antibacterial activity of the synthesized azo compound. The Department of Biotechnology at MPASC College, Panvel-Raigad, India (affiliated with the University of Mumbai), provided two human pathogenic bacterial strains: Staphylococcus aureus (a gram-positive bacterium) and Escherichia

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coli (a gram-negative bacterium). *E. coli* (25922) and S. aureus (23235) are the ATCC numbers of microorganisms. Sterile Muller Hinton Agar Media was used to inoculate all bacterial isolates, which were then incubated at 37°C for 24 hours. The Mueller–Hinton agar plates were streaked with the juvenile bacterial culture. Then, 6 mm wells were cut into the nutrient agar plates to evaluate the antimicrobial activity of the azo compound [8][14]. To observe the formation of the growth inhibition zone around the wells and incubated at 37 °C for 24 hours. The results were recorded. The antibacterial activity of the was compared to that of the standard Gentamicin antibiotic [8][15].

2.6 Antioxidant activity by DPPH method

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was used to assess the antioxidant activity of the dicyanoisophorone azo molecule that was synthesised. The drug was dissolved in a 1 mg/mL stock solution [15], and 2 mL of freshly made 0.2 mM DPPH in methanol was combined with 50 µL of this solution. For 30 minutes, the mixture was left to incubate in a dark place at 37°C. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer after incubation. The positive control was a 1 mM ascorbic acid solution
[16][17]. Antioxidant activity was calculated as the percentage of DPPH radical scavenging using the following equation:

$$\%\text{Inhibition} = \left(\frac{\text{A control} - \text{A sample}}{\text{A control}}\right) X\ 100$$

Where:

$A_{control}$ is the absorbance of the DPPH solution without the antioxidant (control).

A_{sample} is the absorbance of the DPPH solution with the antioxidant.

III. RESULT AND DISCUSSION

3.1 Characterization of synthesized Dicyanoisophorone based azo Compound:

The synthesis of the library of Dicyanoisophorone based azo Compound (Table -1, Fig. 1) was performed according to the method in the literature.**^{1}H NMR** (600 MHz, $CDCl_3$) δ 7.80 (d, 2H), 7.05 (d, 1H), 7.00 (d, 2H), 6.93 (d, 1H), 6.82 (s, 1H), 6.66 (d, 1H), 6.67(d, 1H), 6.42 (s, 1H), 4.35(d, 2H), 2.49 (s, 2H), 2.48 (s, 2H), 1.06 (s, 6H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 169.35, 168.77, 154.92, 148.47, 137.78, 133.78, 129.49, 127.94, 126.03, 125.06, 121.95, 115.09, 113.23, 112.30, 42.09, 39.24, 32.01, 28.04.**HR-MS** calculated for $C_{25}H_{22}N_4O_2$ = 410.48; found (M + H)411.18. The purity of synthesized dicyanoisophorone based azocompound by**HPLC** is 96.40%. **FTIR** spectrophotometry was employed to identify and verify the presence of specific functional groups. The analysis revealed several characteristic stretching vibrations: 1. Azo group (N=N): 1576.92 cm^{-1} (**Figure 2**) 2. Methyl group: 2909.39 cm^{-1} 3. OH group: 3438.72 cm^{-1} 4. CN group: 2202.74 cm^{-1} (**Figure 2**) 5. Aromatic ring: 1670.95 cm^{-1}.

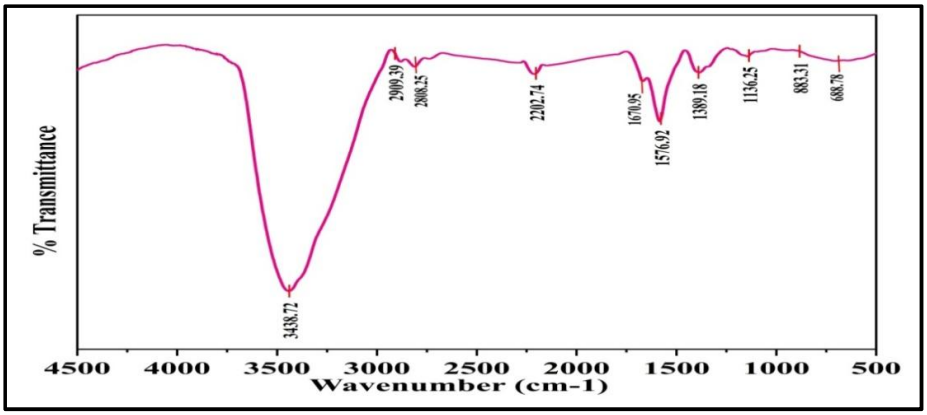

Figure 2. FTIR spectra of Dicyanoisophorone based Azo Compound.

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Table 1. Physical properties of the Dicyanoisophorone based Azo Compound.

Compound	Molecular Formula	Molecular weight (g/mol)	Yield (%)	Melting Point (°C)	R_f value
	$C_{25}H_{22}N_4O_2$	410.48	84	110	0.84

3.2 Antimicrobial Activity of Synthesized Dicyanoisophorone based Azo Compound:

The antibacterial effect of the azo compound primarily results from the disruption of cell membranes in *Staphylococcus aureus* and *Escherichia coli*. Additionally, the compound induces cell elongation by damaging bacterial DNA and generating oxidative stress within the bacterial system[18], ultimately leading to cell death. These cations were more readily absorbed and attached to the surface of *Escherichia coli* compared to *Staphylococcus aureus*[19]. They interacted with proteins and phospholipids on the bacterial surfaces, leading to the leakage of cellular materials, including cytoplasmic contents, from both *Escherichia coli* and *Staphylococcus aureus*. The DNA of the bacterial system experiences significant damage due to oxidative stress. The enhanced antibacterial effectiveness of the Dicyanoisophorone azo compound can be attributed to all of these factors[8].

The Dicyanoisophorone based azo molecule exhibited antibacterial efficacy against Gram-negative as well as Gram-positive bacteria. As illustrated in Fig.3, when tested against Escherichia coli, a Gram-negative microorganism, the azo compound produced a growth-inhibitory zone measuring 14 mm in diameter. This result was comparable to that of the standard antibiotic gentamicin, which exhibited a 15 mm inhibition zone. The better growth-inhibitory zone is recorded in E-coli by azo compound at all concentrations of 25µ (6 mm), 50µ (12 mm), and 100µ (14mm) as compared with standard (14mm). Similarly, for Staphylococcus aureus, a Gram-positive bacterium, the azo compound created a 25µ (14 mm), 50µ (16 mm), 100µ (18mm) mm growth-inhibitory zone, which was nearly equivalent to the 23 mm zone observed with gentamicin (**Figure.3**). These findings suggest that the Dicyanoisophorone-based azo Dye possesses significant antibacterial properties against both types of microorganisms, with efficacy approaching that of a standard antibiotic[20].

Antimicrobial activity results (Table 2) show that the azo compounds were tested against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) using the disk diffusion method. The compounds demonstrated notable antimicrobial effects, particularly against both *S. aureus* and *E. coli*.[21].

Table 2: Antimicrobial activities of Dicyanoisophorone based Azo Compound

Compound	Diameter of zone of inhibition in mm					
	Escherichia coli			*Staphylococcus aureus*		
	25µ	50µ	100µ	25µ	50µ	100µ
Azo Compound	6	12	14	14	16	18
Standard	15			23		

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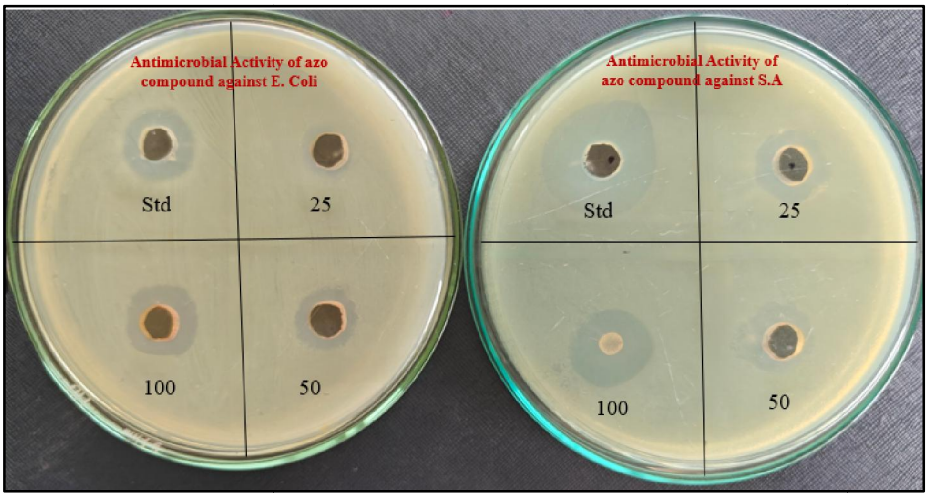

Figure 3. Antibacterial activity of Dicyanoisophorone based Azo Compound against Gram-positive (*S. Aureus*) and Gram-negative (*E. Coli*).

3.4 Antioxidant activity by DPPH method:

Fig.4. display the UV-visible spectra and images of antioxidant activity for the control, standard, and Dicyanoisophorone based azo compounds, respectively. The DPPH solution is used to quantify the antioxidant activity of compounds that can undergo electron transfer.A chromophore is a stable radical cation that appears purple and exhibits maximum absorption at a wavelength of 517 nm[22].When added to a solution containing DPPH radical, antioxidant compounds that can donate an electron to the radical cation cause the solution to decolorizes [23]. Radical scavenging activity is analyzed using Antioxidant activity by DPPH method.A decrease in absorbance and a shift in colour from purple to yellow to colourless indicate a reduction in DPPH radical concentration (**Figure.4**). The absorbance and percentage inhibition of scavenging activity for the azo compound synthesized from dicyanoisophorone are presented in Table 3. The data show that a 50 µg/ml solution of the azo Compound achieves 90.77% inhibition, compared to 96.86% inhibition for the standard ascorbic acid. This indicates that the azo compound exhibits significant antioxidant properties [24][22].

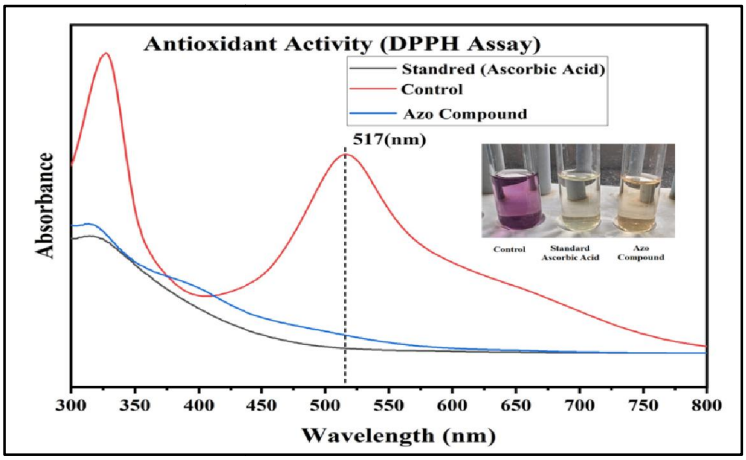

Figure 4.UV-visible spectra for antioxidant activity of Dicyanoisophorone based azo Compound by DPPH scavenging method against std. ascorbic acid

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Table 3.Percentage inhibition calculation for antioxidant activity of the Dicyanoisophorone based azo compound.

Sample	Absorbance	% of inhibition
Control (DPPH solution)	0.607	--
Standard (Ascorbic acid) (50ug/mL)	0.019	96.86%
Azo Compound	0.056	90.77%

IV. CONCLUSION

In conclusion, we have demonstrated the synthesis and characterization of new Dicyanoisophorone based azo compound which showed significant antimicrobial and antioxidant properties. The antibacterial effect of the azo compound primarily stems from its ability to disrupt cell membranes in both Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria, with inhibition zones comparable to those of the standard antibiotic gentamicin. The antibacterial mechanism involves disrupting cell membranes, causing DNA damage, and inducing oxidative stress, ultimately leading to cell death. The antimicrobial efficacy of the azo compound was particularly notable against *S. aureus* and *E. coli*. The azo compound showed particularly noteworthy antimicrobial efficacy against *S. aureus* and *E. coli*, with growth-inhibitory zones measuring 18 mm and 14 mm, respectively, at 100 µg concentrations. These values are comparable to those observed for the standard antibiotic gentamicin, highlighting the compound's potential as an effective antimicrobial agent against both Gram-positive and Gram-negative bacteria. The azo compound also exhibits notable antioxidant properties, as evidenced by its ability to scavenge DPPH radicals. At a concentration of 50 µg/ml, the azo compound achieved 90.77% inhibition, which is substantial compared to the 96.86% inhibition of the standard ascorbic acid. This antioxidant activity is attributed to the azo compound's electron-donating capability, resulting in the decolorisation of the DPPH solution. These findings highlight the potential of dicyanoisophorone azo compound as a multifunctional compound with both antimicrobial and antioxidant properties. Further research on applications of the synthesized compound in the suitable fields is currently ongoing in our laboratory and results will be published soon in elsewhere.

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Credit Authorship Contributions Statement

Kajal R. Gaikwad: Conceptualization, synthesis, characterization, antimicrobial and antioxidant studies, writing-original draft preparation.
Aparna A. Dhumal: Biological analysis validation, antimicrobial assays, editing of manuscript.
Pramod B. Thakur: Supervision, project administration, methodology guidance, writing-review and editing.

Declaration of Competing Interest

The author declares no conflict of interest, financial or personal, that could have influenced the work reported in this paper.

Consent to Publish Declaration

Not applicable.

Ethics and Consent to Participate Declarations

Not applicable.

Data Availability:

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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