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Prodrugs: A Comparison of *o*-Nitrobenzyl, *p*-Hydroxyphenacyl, and Coumarin Photoremovable Protecting Groups and Their *In Vivo* Applications in Cancer Therapy

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Abstract

Many of the anticancer drugs currently used face significant drawbacks, including poor site specificity and a lack of targeted treatment. As a result, during chemotherapy and other procedures, healthy cells and tissues in the body are damaged in addition to cancer cells. Prodrugs are therapeutic agents designed to improve the pharmacokinetic properties of a drug with the attachment of a protecting group. The drug is then chemically activated *in vivo* through varying cleavage mechanisms, thus providing more accurate and selective treatment for the given disease. There has been a steady increase in the implementation of prodrugs in recent years, with 5-7% of the total approved medicines worldwide and approximately 15% of all new medicines approved each year falling under that category. One favorable pathway of activation is photostimulation, or irradiation of the prodrug with specific wavelengths of light. The most commonly used photoremovable protecting group (PPG) is *o*-nitrobenzyl (oNB) due to its versatility and ability to successfully activate a variety of functional groups. However, recent studies center on the development of newer protecting groups that can overcome nitrobenzyl's limitation of a slow release rate. Among these are *p*-hydroxyphenacyl (pHP) and coumarin, which have rapid reaction rates that make them suitable for tracking the kinetics of biological processes. Since these protecting groups are being tested under different conditions and on different drugs, it is important to develop a standard method of measuring successful activation. Through a review of previous research studies, this paper seeks to compare the three photosensitive protecting groups and discuss their advantages and disadvantages. In turn, it determines the most effective treatment for the targeting of various biological applications, ranging from neurotransmission to cancer chemotherapy.

Keywords

Prodrugs, Therapeutic Agents, Photosimulation, Protecting Group, Anticancer, UV Radiation

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Introduction

Prodrugs are biologically inactive compounds converted into their active form of medication *in vivo*, allowing them to overcome various barriers in the drug delivery process. These barriers are classified together as ADMET (absorption, distribution, metabolism, excretion, and toxicity), but more specifically, they may include limited aqueous solubility, as seen in corticosteroids, or poor site specificity, as seen in anticancer agents (2). Within a prodrug, the pharmacological activity of the drug is masked (caged) and requires chemical activation to trigger structural rearrangement and release the active drug *in vivo* (Figure 1).

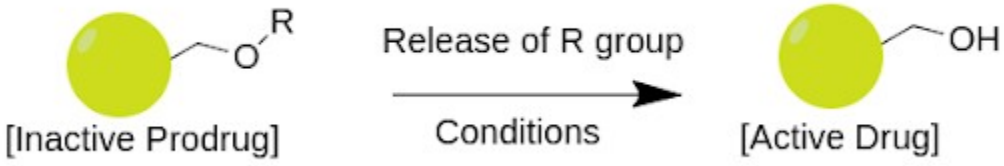

Figure 1: General schematic for prodrug strategy wherein a drug molecule can be chemically modified through the attachment of a functional group R. This creates a prodrug which can then be released under controlled conditions to release the active drug.

Removal of the promoiety, or protecting group, can be achieved through enzymatic reaction or exposure to a certain pH (with the addition of acids or bases to the cells), but the most promising pathway for activation is found to be photostimulation (3). In the case of enzymatic reaction, enzymes may exhibit issues of biodegradability due to their sensitive nature in the specific conditions for cleavage, or inability to cross an impermeable membrane (4). As regards pH cleavage, most of the protecting groups or linkers so far have been developed *in vitro*, and thus require in depth *in vivo* research before the novel drugs undergo clinical trials (5). As a result, photoremovable protecting groups (PPGs) as a method for targeted drug delivery have gained the most attention and advancement. These linkers can be synthesized and chemically modified onto the parent drug, then irradiated with a certain wavelength of light. Currently, light in the UV range (100-400 nm) is utilized, which presents a major limitation with regard to its applications *in vivo* due to the absorption of UV radiation by the human body. This results in not only reduced effectiveness of the cleavage process arising from poor penetration depth, but also causes damage to biological tissues, which may lead to iatrogenic carcinogeneis. Current research aims to address this through the development

of photosensitive linkers that can be cleaved at alternative wavelength ranges, including visible light or infrared. This involves the testing of a variety of electron donating and/or electron withdrawing functional groups to be chemically attached to the parent drug (6). The purpose of this paper is to compare the efficacy of various photoremovable protecting groups in photocleavage, including *o*-nitrobenzyl (oNB), *p*-hydroxyphenacyl (pHP), and coumarin, as well as their accuracy in targeting cancer cells *in vivo*. Significant characteristics to consider include ease of synthesis, toxicity or adverse effects to the body, yield of the active drug, aqueous solubility, and chemical stability.

_o_-Nitrobenzyl (oNB)

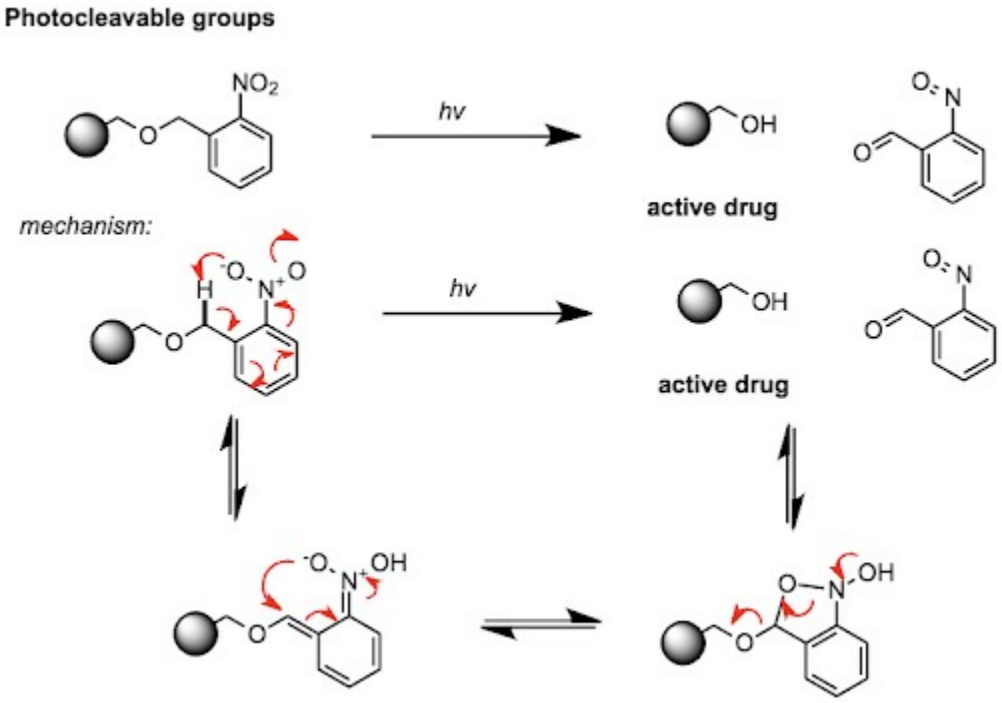

Figure 2: Mechanism for the photocleavage of nitrobenzyl (oNB) upon irradiation with UV light. R represents the varying functional group attached to the benzylic site of the protecting group.

The *o*-nitrobenzyl group and its compounds are among the most commonly used PPGs due to their ability to derivatize a large variety of functional groups (Figure 2). These include carboxylic acids (R−COOH), thiols (R−SH), phosphates (PO_4^{3-}), carbamates (H_2NCOO^-), and carbonates (CO_3^{2-}), which are all leaving groups that can be directly attached to the benzylic site of the PPG (7). Given its advantages of straightforward synthesis of the caged promoiety, reliable activation under varying experimental systems, and high-

resolution spatial control of reactions, the *o*-nitrobenzyl group has been used for numerous biological applications, particularly the combinatorial synthesis of biopolymer microarrays. This process of photolithography can yield nucleic acid arrays with $>10^6$ unique sequences of DNA or RNA per square centimeter in the span of a few hours (8). With this large sequence space, microarrays are ideal platforms for exploring the mechanisms of nucleic acid ligand interactions and constructing retrievable reservoirs of information (9). A study conducted by Kretschy *et al*. prepared a derivative of the nitrobenzyl group, 2-(2-nitrophenyl)propoxy- carbonyl (NPPOC), in MeOH and 365 nm irradiated light. They observed a relatively high photolysis quantum yield (0.41) that enabled the creation of microarrays of long oligonucleotides (8,10). However, its low molar absorptivity ($230\ M^{-1}cm^{-1}$) limits the application of the protecting group at larger wavelengths of light (8). This has prompted a search for groups with higher absorptivity. Jullien et. al. studied potential modifications of the appended functional groups on the prodrug in an attempt to improve the efficiency of *o*-nitrobenzyl. After synthesis of the derivative 4,5-di- methoxy-2-nitrobenzyl and the attachment of electron-withdrawing substituents at the benzylic site, they found that the quantum efficiency, a measure of the amount of electrons emitted in relation to the photons of the energy incident, did not increase (11). In contrast, an alternate study done by Pirrung demonstrated that the deprotection of the compound with this modification was quite efficient (12). The contradiction of the results may stem from differing experimental parameters and the discrepancy in multiple variables, including but not limited, to irradiation wavelength (254 nm vs 350 nm), solvent (methanol vs acetonitrile), and leaving groups (pentadienyl vs ethyl cinnamate). These differences have a large impact on cleavage, solvation, and proton transfer, which can significantly alter the reaction mechanism.

The oNB protecting group has been tested on drugs such as serotonin, which is a neurotransmitter used as a therapeutic agent to target mood disorders. A long wavelength was required to activate the prodrug and avoid complications such as cell damage or additional photochemical side reactions. However, photolysis at this wavelength (laser light of 308 nm), led to a lower quantum yield, represented by the number of serotonin molecules released, which is a measure for the success of the protecting group. At shorter wavelengths, damage to the NIE-115 cells used in the study occurred (13). A potential solution to address this issue is the use of conjugation to increase the maximum absorption to longer wavelengths. Thus, the prodrug can be irradiated with visible light (380 nm - 700 nm)

or IR radiation (780 nm - 1 mm) rather than UV light (100 - 400 nm), which would limit the body's absorption and thus prevent cell damage. This can be accomplished through conjugation addition reactions, which append a double bond on a nucleophile to a carbonyl group (14). The application of the oNB group should be carefully considered because of the further disadvantages it demonstrates, including a slow reaction and thus unsuitability to rapid release. This is most prominently an issue in cases of electrophysiological applications, such as electrical cardioversion. Additionally, oNB may undergo other side reactions, forming potentially toxic byproducts such as o-nitrobenzaldehyde, that absorb the incident light and create a filter that inhibits successful activation (7,12). Future studies should seek to determine modifications that can address these issues and to consider alternate protecting groups that can be applied.

_p_-Hydroxyphenacyl (pHP)

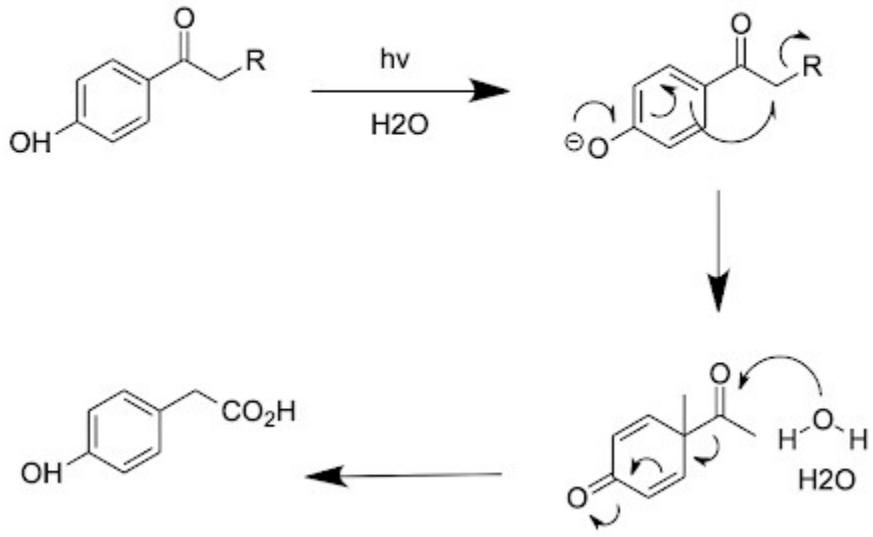

Figure 3: Mechanism for the photohydrolysis of the p-hydroxyphenacyl (pHP) substrate bond from the triplet phenol upon irradiation with UV light. One possible byproduct of this reaction includes the appearance of < 10% of 2,4-dihydroxyacetophenone.

One of the newer developed photoremovable protecting groups is the *p*-hydroxyphenacyl (pHP) group, which is primarily attached to carboxylic acids (R−COOH) and hydroxyls (-OH) and can act as a cage for bioactive substrates (Figure 3). It has several promising

properties, including efficient photochemical release ($\Phi \approx 0.10–1.0$), high quantum yields, clean reactions (only yields one significant byproduct without setting off secondary photoreactions), and good aqueous solubility or hydrophilicity, which is especially vital for biological applications. Ideally, the PPG would have reasonable absorptivity at irradiation wavelengths greater than 300 nm, so as to prevent harmful UV radiation (15). Although pHP has weak absorptivity above 300 nm, this can be remedied by modifying the aromatic substituents through the addition of electron donating methoxy ($O\text{-}CH_3$) or carboxyl ($R{-}COOH$) groups at the ortho or meta directors (7). This in turn increases the versatility of the pHP group by allowing it to absorb light at longer wavelengths (approaching the visible range > 375 nm) and improving solubility (16). However, the substituent also has a significant effect on the quantum efficiency of release. Despite its advantage of operability at increased wavelengths, electron-donating groups such as $3\text{-}OCH_3$ displayed lower quantum yields compared to electron- withdrawing groups such as 3-CN. Overall, the rate constants for release remained consistently high, at around 10^9 s^{-1} (15). Consequently, further investigation is required to determine the functional group with the best balance of wavelength and quantum efficiency.

The rapid release rates achieved by the pHP protecting group allows it to be primarily applied to the rapid events in neuro-transmission and signal transduction. For instance, pHP-I-Glu is a caged derivative of glutamate (Glu), an excitatory neuro-transmitter responsible for sending signals between nerve cells as well as controlling learning and memory in the brain. A study conducted by Kandler used pHP-I-Glu to study postsynaptic long-term depression (LTD) and its influence on chemical synaptic transmission in isolated mice brain cells, specifically CA1 hippocampal pyramidal cells. The study found that rapid glutamatergic currents were generated by the photorelease of glutamate, therefore demonstrating successful cleavage. However, one drawback observed is the short duration of the pulses (< 50 ms), which resulted in a low concentration of free glutamate (< 50–200 μM) that in turn limited NMDA receptor activation and triggered LTD (15,16). This drawback may require the modification of the receptors or introduce a greater number of them in order to overcome the decrease in responsiveness contributing to LTD.

Coumarin

The coumarin protecting group is primarily used for the photorelease of leaving groups such as phosphates (PO_4^{3-}) and sulfonates (R-

SO_3^-) (Figure 4). Due to its advantages of fast release rates, improved derivative stability, and low toxicity, it has received increased attention in recent years as a potential application in photochemotherapy and alternative cancer treatments (7). Coumarin's ability to counter-act the harmful side effects caused by radiotherapy have spurred investigations of the protecting group in the treatment of prostate cancer, renal cell carcinoma, and leukemia (17). Additionally, coumarin's large molar absorption at irradiation with longer wavelengths (extending into the visible region, 400-500 nm) provides an advantage over the previous protecting groups discussed, oNB and pHP, and its fluorescent properties, which these groups do not possess, also allow for convenient monitoring of the reaction progression (18). Initially, coumarin experienced several limitations including low water solubility, marginal quantum yields, and low absorption maximum, but these were addressed through modifications of the substituents at C6 and C7 into alkoxy and amino groups respectively. This shifted the absorption maximum by 30 nm, into the 350-400 nm range, and recorded the highest quantum yields out of all the analogues (7). The problem of low solubility could be improved by appending hydrophilic polar groups such as carboxylates ($RCOO^-$), because water is a polar solvent, and it will dissolve polar groups. The synthesis and addition of polyaromatic analogues were also synthesized to improve the fluorescent properties of coumarin and its use as a tag (7,18).

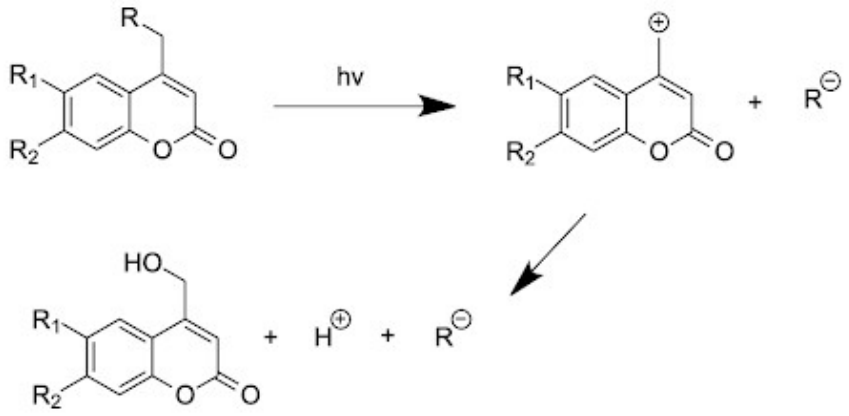

Figure 4: Mechanism for the photocleavage of coumarin upon irradiation with 350 nm UV light. R1 and R2 represent alternate potential functional groups on the prodrug whereas R represents the primary leaving group.

One study conducted by Hayashi attempted to mask the pharmacological activity of paclitaxel, an important drug used in cancer chemotherapy to treat ovarian, breast, and lung cancers (19). However, this agent possesses

very little to no specificity for the target tumor tissues, leading to toxicity and damage to healthy tissues in the body. Thus, coumarin was conjugated onto the benzylamine group and then irradiated with visible light (430 nm), which resulted in selective activation of the prodrug phototaxel and conversion into the parent bioactive molecule, paclitaxel, through spontaneous *O*-*N* intramolecular acyl migration reaction. A yield of approximately 69% was observed using HPLC (High-performance liquid chromatography) analysis, which was in agreement with the typical experimental yield of 40-70%. Diethylamino-4-hydroxymethyl coumarin was chosen as the photosensitive group due to its solubility in water, thermal stability, and rapid photolysis rate by irradiation with visible light discovered from previous studies (20). However, one notable issue was the low water solubility of paclitaxel, especially in the intravenous mileau. A longer administration time was required to inject the drug, but because of this, the detergent that solubilizes paclitaxel, Cremophor® EL, produced harmful side effects that required treatment with additional medication, corticosteroids and antihistamines (19). All of this can greatly burden the patient's immune system, which is already in a severely weakened state. However, overall, the favorable results of this study suggest that the coumarin protecting group has the potential to be applicable to a wide range of anticancer agents for the development of new photocleavable prodrugs (20).

Conclusion

Photoremovable protecting groups are synthesized and deployed for a wide range of biological applications, each possessing their own set of advantages and disadvantages. This paper elucidates some of their novel case studies, but much remains to be learned regarding their general implementation on active drugs in order to effectively serve as treatments. Currently, *o*-nitrobenzyl (oNB) is the most commonly and widely used protecting group due to its versatility, as it can protect a variety of different functional groups and activate in varying experimental conditions (e.g. both in solution and in the solid state) (7,15). However, as research on prodrugs increases, studies are observing newer, more efficient protecting groups that can replace the commonly used *o*-nitrobenzyl group. For instance, *p*-hydroxyphenacyl (pHP) has been successfully applied to biological processes that take advantage of its rapid release rate and clean reactions, including enzyme catalysis (substrate binding and enzyme conformational changes), dynamic inhibition, and the tracking of signal transduction kinetics (21). Similarly, coumarin has fast release rates as well as the added benefits of hydrolytic stability and low toxicity that make it ideal for applications in

cancer therapy as well as in studies involving the rapid release of nucleosides and nucleotides such as ATP and GTP. Other photoremovable protecting groups have also in fact been studied and industrially applied, such as benzoin and aryl ketones (7,22,23). However, because their advantages are similar to the groups already mentioned in this review paper, they are not discussed here. Future studies should aim to determine the adaptability of the protecting groups in varying experimental parameters, which is the primary advantage for *o*-nitrobenzyl, by changing variables such as pH, temperature, irradiated wavelength, etc. Additionally, experiments should be performed with conjugation as a means of addressing the issue of UV damage at irradiation with shorter wavelengths. By resolving these limitations and comparing the advantages of different photoremovable protecting groups, it will be easier to determine their specific applications in the human body for more efficient and targeted treatment.

Table 1: Comparison of the three photoremovable protecting groups discussed along with various attached substituents, irradiation wavelengths, solvents, and resulting quantum yields.

	R (leaving group)	**λ_{max} / nm**	**Solvent**	***Φ***
***o*-Nitrobenzyl (oNB)**	CH_3CO_2	254	MeCN	0.20
	4,5-dimethoxy	305	Et_3N	0.16
	sugar-O	364	Buffer, pH 7	0.62
	RCO_2	370	MeCN/H2O, 3:2	0.080-0.16
***p*-Hydroxyphenacyl (pHP)**	CH_3CO_2	279	H2O (10% - 50%)	0.40
	Glu	273	H2O (10% - 50%)	0.14
	OPO_3Et_2	271	H2O (10% - 50%)	0.40
	ATP	320	H2O (10% - 50%)	0.37
Coumarin	OMe (R_1) H (R_2)	340	HEK extracellular buffer	0.020
	Glu (R_1) NEt_2 (R_2)	400	n/a	0.11
	H (R_1) NMe_2 (R_2)	365	HEK293 cells	0.26
	H (R_1) NEt_2 (R_2)	365	HEK293 cells	0.23

Data adapted from Klan P. et al. (2013); Givens R. et al. (2012).

Table 2: Comparison of various structural categories of anticancer drugs and their most effective photoremovable protecting group pairing.

Category of Drug	**Leaving Group pKa**	***Φ***	**Release Yield**	**Recommended PPG**	**References**
Alkylating Agents (EX: Chlorambucil)	5.80	0.46	90%	*o*-Nitrobenzyl (oNB)	26
Neurotransmitters (EX: GABA)	4.76	0.21	87%	*p*-Hydroxyphenacyl (pHP)	7
Bioactive phosphates (EX: ATP and GTP)	7.40	0.37	80 - 90%	*p*-Hydroxyphenacyl (pHP)	7
Kinase inhibitors	5.80	0.21	85 - 90%	*o*-Nitrobenzyl (oNB)	21
Antibiotics	9.40	0.25	> 90%	Coumarin	25
Antimicrotubule agents (EX: Paclitaxel)	10.4	0.07	89%	Coumarin	20
Alkaloids	7.20	0.04	90%	*p*-Hydroxyphenacyl (pHP)	24
Anthracyclines (EX: Doxorubicin)	9.53	0.08	88%	Coumarin	17

Table 3: Summary of the various advantages and disadvantages of the three photoremovable protecting groups as well as potential modifications to address their drawbacks.

	***o*-Nitrobenzyl (oNB)**	***p*-Hydroxyphenacyl (pHP)**	**Coumarin**
Functional Groups	- carboxylic acids (R−COOH) - thiols (R−SH) - phosphates (PO_4^{3-}) - carbamates (H_2NCOO^-) - carbonates (CO_3^{2-})	- carboxylic acids (R−COOH) - hydroxyls (-OH)	- phosphates (PO_4^{3-}) - sulfonates ($R\text{-}SO_3^-$)
Advantages	- easy synthesis reaction - reliable activation in varying experimental conditions - can derivative a variety of functional groups	- rapid release rate - high quantum yields - clean reactions (no secondary byproducts) - good aqueous solubility	- rapid release rate - improved derivative stability - low toxicity - high molar absorptivity at long wavelengths (400-500 nm) - fluorescent properties
Disadvantages	- low molar absorptivity at long wavelengths - slow reaction - side reactions create toxic byproducts	- low molar absorptivity above 300 nm	- low water solubility - marginal quantum yields
Potential Modifications	Conjugation addition reactions to shift the maximum absorption to longer wavelengths	Modification of aromatic subsitutents through the addition of electron donating groups. This will allow the PPG to absorb light at longer wavelengths (approaching the visible range > 375 nm) as well as improve solubility	Appendage of hydrophilic polar groups such as carboxylates ($RCOO^-$). Since water is a polar solvent, it will dissolve polar groups and thus improve solubility

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