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1. ...ENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	12/09/91	Final Report 89-04-15-92:04:14

4. TITLE AND SUBTITLE
Conformation of Membrane Proteins: Bacteriorhodopsin

5. FUNDING NUMBERS
Contract # DAAL03-89-K-0088

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8. PERFORMING ORGANIZATION REPORT NUMBER

DTIC ELECTE APR 13 1992 S D D

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

...PONSORING/MONITORING ...GENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
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12a. DISTRIBUTION/AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

One of the main goals of this research project was accomplished. A water soluble derivative of bacteriorhodopsin (BR) was synthesized. An activated species of methoxypolyethylene glycol (MeOPEG) was synthesized, yielding 2-0-methoxypolyethylene glycol-N-hydroxy succinimyl carbonate. (MeO-PEG-SC). MeO-PEG-SC was coupled with the purple membrane (PM) of Halobacterium halobium to yield MeO-PEG-PM. This product was centrifuged and purified by washing with H_20 (76% conversion), dissolved in buffer with 5% sodium dodecylsulphate (SDS), (1:1) and PAGE Electrophoresis performed. The separated MeO-PEG-BR band was recovered from the gel by electroelution, and the solution lyophilized. This material was dissolved in H_20 and the SDS removed by passing the solution through an Extra-gel column (Pierce) and eluted with PO_4 buffer pH 7.0. The final solution was centrifuged at 200,000 g, yielding a clear water-soluble solution of MeO-PEG-BR. The product could be reconstituted into miscells which were capable of proton pumping.

The MeO-PEG-BR reconstituted into vesicles had physical-chemical properties identical with the original PM. The circular dichroism spectra, ultraviolet and visible spectrum, and fluorescence spectrum were identical to that of the PM.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Proteins, Membranes, Conformation, Bacteriorhodopsin	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

CONFORMATION OF MEMBRANE PROTEINS:

BACTERIORHODOPSIN

GERALD D. FASMAN

U. S. ARMY RESEARCH OFFICE

CONTRACT # DAAL03-89-K-0088

BRANDEIS UNIVERSITY

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Technical Report: Final 1991

Summary of Results

The majority of the objectives outlined, in the initial proposal, for the first 24-month period, have been achieved. A methoxy-polyethylene glycol (MeO-PEG) derivative of bacteriorhodopsin (BR) (MeO-PEG-BR) was synthesized, isolated, and purified. A thorough investigation, using five different activated species of MeO-PEG-X, was carried out to obtain a maximum yield of the MeO-PEG-BR conjugate. The most satisfactory yield was obtained using 2-0-methoxypolyethylene glycol succinimdyl carbonate. The yield could be maximized by having an excess of methoxypolyethylene glycol present during coupling. The MeO-PEG-BR was successfully isolated, and refolded into micelles and vesicles. The refolded conjugate had a native-like structure, as shown by UV-Vis, CD and fluorescence spectroscopy and by the fact that proton pumping was demonstrated. Stable intermediates produced during unfolding were obtained by the addition of denaturants. A reversibly formed transient intermediate with an absorbance maximum of ≈ 480-510 nm was also found.

RESULTS

1. The growth of the Halobacterium halobium was optimized (e.g. aeration rate, light intensity, etc.) and the purple membrane (PM) can now easily be obtained in sufficient yields to carry out the desired experiments.

2. Synthesis of Activated MeO-Polyethylene glycol (MeO-PEG-OH) and MeO-Polyethlene glycol-bacteriorhodopsin (MeO-PEG-BR).

   The first major goal of this research project was to investigate the folding of bacteriorhodopsin (BR) as it attains its final conformation as it is inserted into the membrane. To investigate the pathway of folding, it was necessary to synthesize a water-soluble derivative of BR. The MeO-PEG-derivative of BR (MeO-PEG-BR) was chosen, as it was known that MeO-PEG-X was an excellent water-stabilizing agent (1). It was necessary to synthesize an active species of MeO-PEG-OH, namely, {MeO-$(OCH_2)_X$ - Y (MeO-PEG-Y), X = 5000, Y = active species} to couple the MeO-PEG to BR. Five different species were tried until a satisfactory yield of product was obtained. These were:

   A. 2-0-methoxy-polyethylene glycol-4,6-dichloro-S-triazine; (2)

B. 2,4-bis-O-methoxypolyethylene glycol-6-chloro-S-triazine; (2)

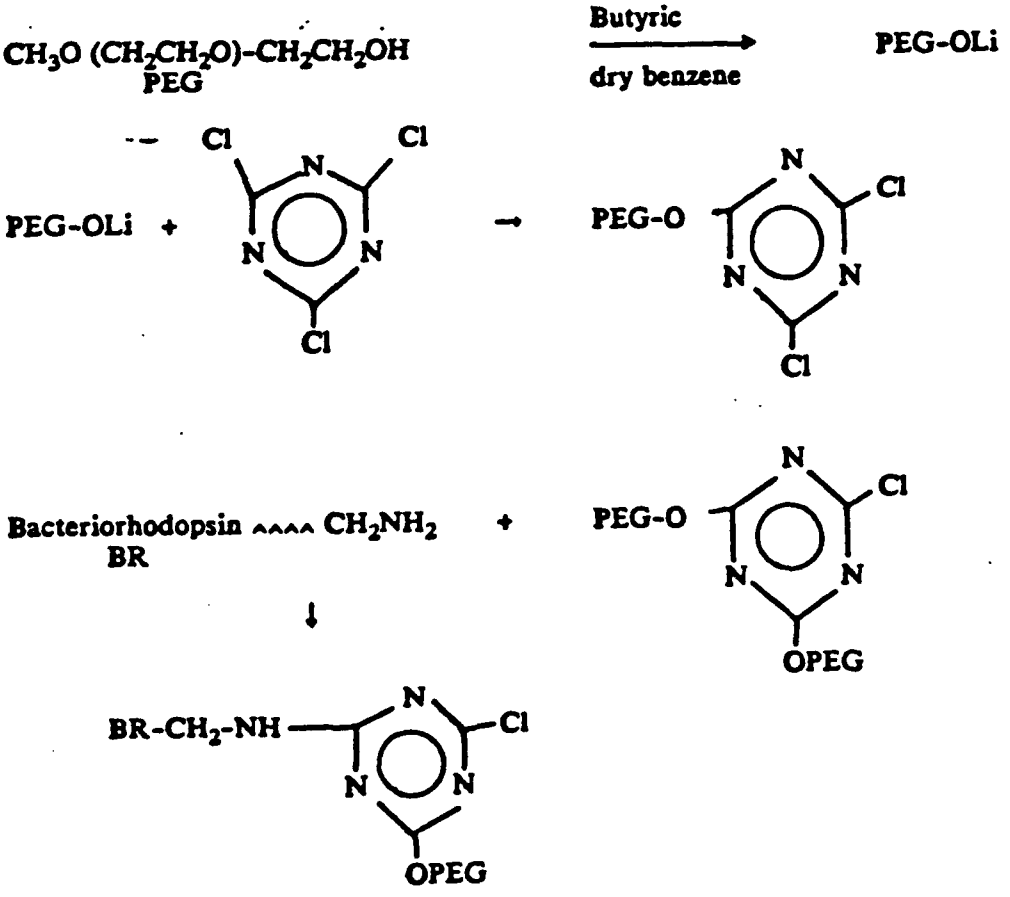
C. 2-O-methoxypolyethylene glycol-succinimidyl-succinate; (3)

D. 2-O-methoxypolyethylene glycol tresylate; (4), and E.2-O-methoxypolyethylene glycol-N-hydroxy succinimidyl carbonate; (4) and

E. 2-O-methoxypolyethylene glycol-N-hydroxy succinimidyl carbonate. (5)

The synthesis of the A and E active species was achieved by the following procedures:

A. 2-O-methoxypolyethylene glycol-4,6-dichloro-S-triazine. This modifying agent was synthesized as follows, and its reaction with BR is shown:



The final conditions used were a 1000-fold excess of coupling agent, added to a suspension of purple membrane (80 μg/ml in a moderately alkaline buffer (sodium tetraborate, 0.1M, pH9.3) The product was dialysed to remove excess hydrolyzed PEG-coupling agent and dissolved in H_2O. The yield of product was 22%, as evaluated from OD_{560}.

The purity of the starting PM samples, and the MeO-PEG-BR product were checked by using the following analytical methods: sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE); ultraviolet and visible (Vis) spectroscopy; circular dichroism (CD) and fluorescence spectroscopy.

The polyethylene glycol derivative (MeO-PEG-BR) of PM, which was obtained by reaction with (2-O-methoxypolyethylene glycol-4,6-dichloro-S-triazine, was also examined by the above methodologies. The UV and Vis spectrum were identical to the unreacted PM (Figs. 1-4), thus indicating that the modification did not alter the conformation of the PM.

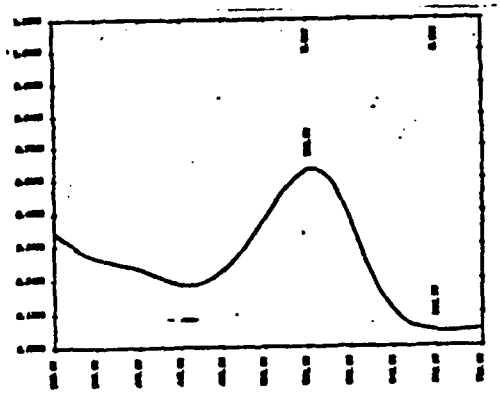


Fig. 1
Vis spectrum of PM

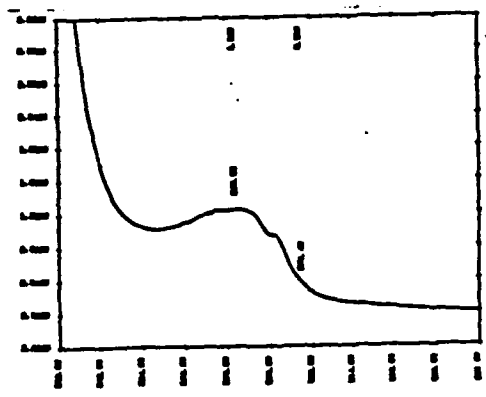


Fig. 2
UV spectrum of PM

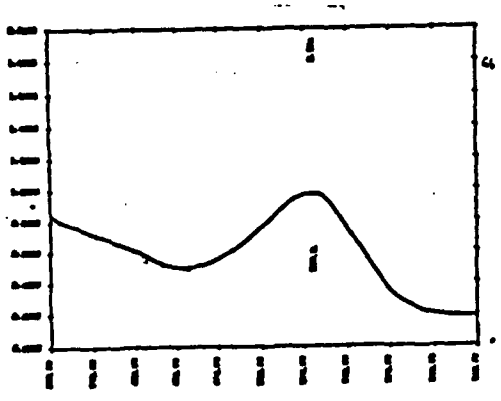


Fig. 3
Vis spectrum of PEG-BR

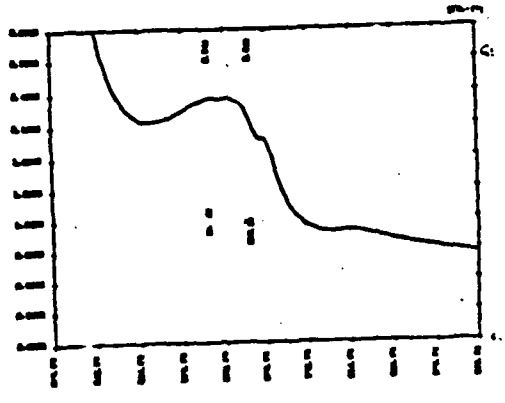


Fig. 4
UV spectrum of PEG-BR

The circular dichroism spectra of the PM and MeO-PEG-BR were also identical (Figs. 5 & 6), giving further evidence that their conformations were identical.

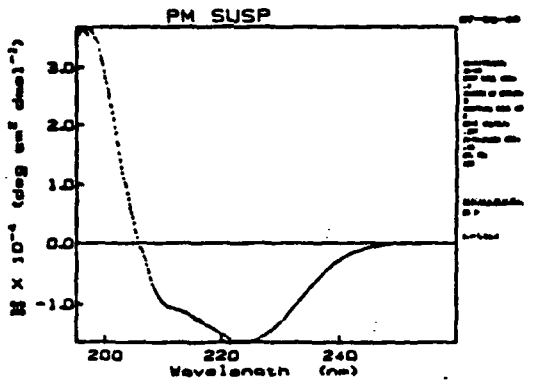


Fig. 5
UV-CD spectrum of PM suspended in H_2O

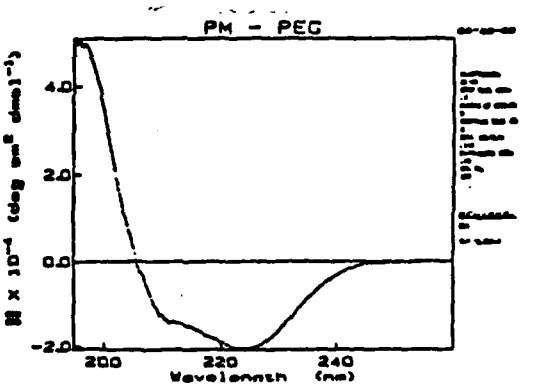


Fig. 6
UV-CD spectrum of PEG-BR in H_2O

Similar fluorescence spectra were obtained:

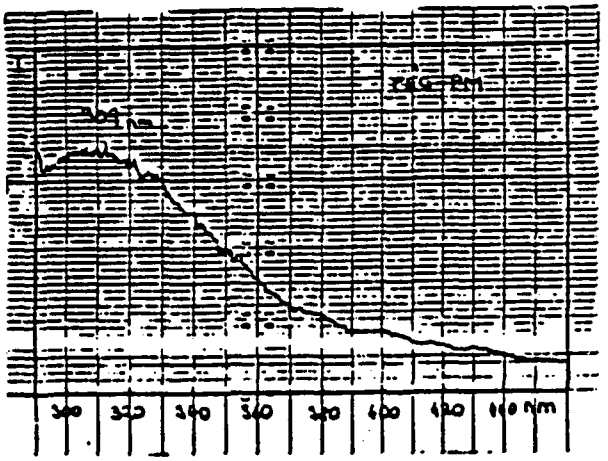


Fig. 11 Fluorescence Spectrum of MeO-PEG-PM

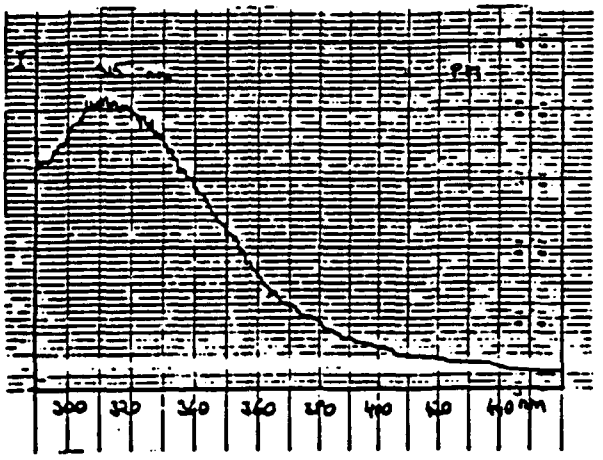


Fig. 12 Fluorescence Spectrum of Purple Membrane

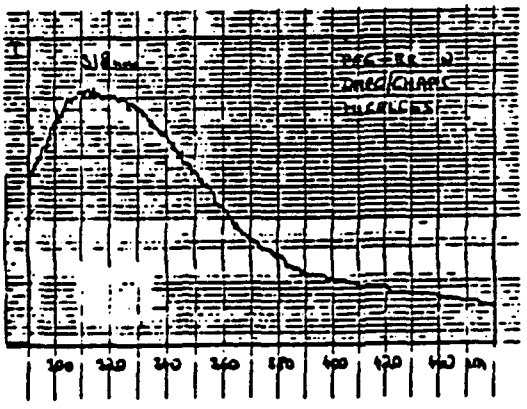


Fig. 13 Fluorescence Spectrum of MeO-PEG-BR in DMPC/CHAPS Miscelles

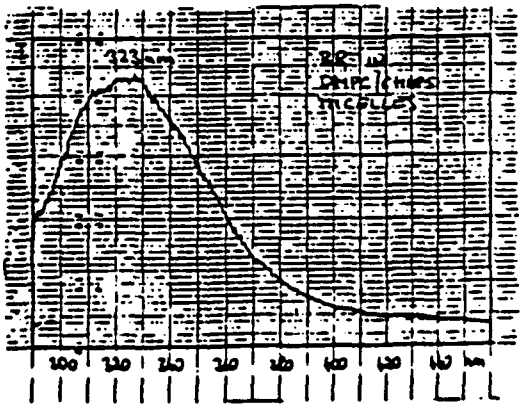


Fig. 14 Fluorescence Spectrum of BR in DMPC/CHAPS Miscelles

The proof that the reaction of PM, plus the activated MeO-PEG-X had occurred, was given by the SDS-PAGE results (Fig. 7).

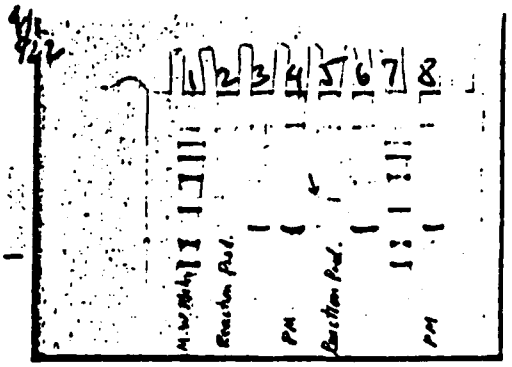


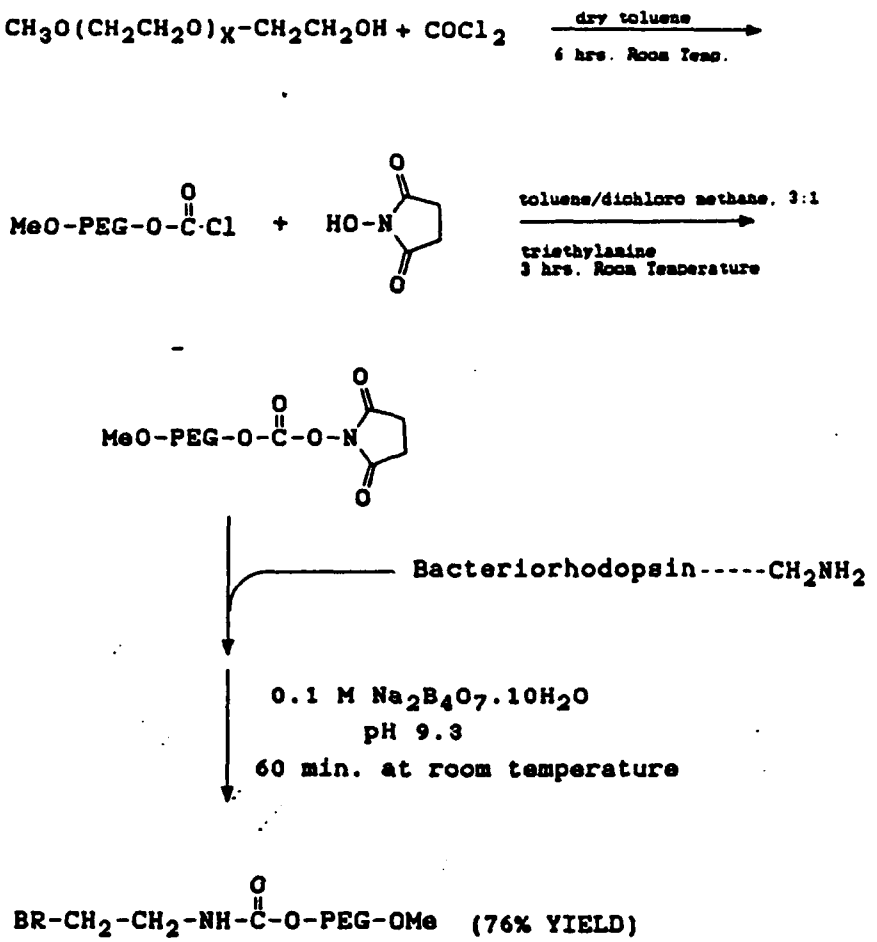
Fig. 7
SDS-PAGE of reaction mixture

The arrow indicates the MeO-PEG-BR derivative.

Five different activated PEG reagents were investigated to obtain a maximum yield of the product, MeO-PEG-BR. They were: 2-0-methoxypolyethylene glycol-4-6-dichloro-S-triazine (1); 2,4-bis-0-methoxypolyethylene glycol-6-chloro-s-triazine (2); 2-0-methoxypolyethylene glycol-succinimidyl succinate (3); 2-0-methoxypolyethylene glycol tresylate (4); and methoxypolyethylene glycol N-hydroxysuccinimidyl carbonate (MeO-PEG-SC).

The yields of the products, and side reactions, were estimated by a gel scan of the SDS-PAGE gel after electrophoresis.

With the use of the first four active species, a large amount of aggregated material remained in the well at the top of the gel upon electrophoresis. HPLC analysis of the sample of Aldrich MeOPEG, used to make the reactive species, showed that a 23% of impurity was present. A new batch of MeOPEG-OH (MW 5000) was obtained from Fluka, which only had a 3% impurity. It was assumed that the aggregation was probably due to the impurity. Therefore, a new batch of coupling agent was synthesized using the Fluka MeOPEG-OH to yield a new activating agent. A new reagent was produced; the synthesis of 2-0-methoxypolyethylene glycol-N-hydroxysuccinimidyl carbonate (MeOPEG-SC), and its coupling to PM, is shown below:



The yield was 85%, and the active carbonate content was ≈ 90%, as determined by reacting aliquots of MeO-PEG-SC with excess benzylamine and back titration of the latter with 0.1M perchloric acid in glacial acetic acid.

The 2-O-MeOPEG-SC-BR was reacted with PM; the coupled membrane was isolated by centrifugation at 1800 rpm and purified by washing with water. The conversion of the PM was 76%. There was a substantial decrease in the amount of aggregated material upon SDS-PAGE analysis, and a high yield of conjugate with a molecular weight of ≈ 33 KDa was obtained.

3. Isolation of MeO-PEG-SC-BR

Upon SDS-Page electrophoresis on 1.5 mm thick, 16 cm x 18 cm gel, the 33 KDa band was cut out, and the MeO-PEG-SC-BR was recovered by electroelution in a BioRad Model 422 ElectroEluter. A 35% yield recovery was obtained, which hopefully can be improved on further studies. The present overall yield of conjugate is 15%, which is reasonable.

The MeO-PEG-SC-BR was obtained as the SDS: MeO-PEG-SC-BR,

1:1, conjugate. Initial attempts at removal of the SDS on an Extra-gel column (Pierce) indicated that this method could be successful with further research.

4. Denaturation Experiments

Unfolding of BR in the purple membrane was attempted by the addition of urea, methylurea, diethylurea, butylurea, and tetramethylurea (TMU). TMU was found to affect the protein conformation reversibly, as detected by visible spectroscopy. In a mixture of 35% v/v TMU in water, the absorbance maximum of PM shifted to 480-510 nm in total darkness. (Native PM absorbs at 560 nm.) The absorbance maximum shifted from 480-510 nm to 380 nm rapidly, upon exposure to light. The rate of the transition was found to be dependent on the TMU concentration, as well as on the intensity of illumination. Dilution of the protein solution, absorbing at 380 nm, with water, to a TMU concentration of 12% v/v, resulted in reversing the spectral changes. The absorbance maximum shifted back to 480-510 nm, then to 560 nm within ≈ 14 days of incubation at room temperature. The presence of the intermediates were detectable even after 20 days.

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