Photochemical Behavior of Limettin

Sang Chul Shim
Department of Chemistry, The Korea Advanced Institute of Science
Seoul, Korea

C_4-Photocycloaddition reactions of limettin, a naturally occurring photodynamic coumarin derivative in plants of the families *Umbilliferae* and *Rutaceae*, are studied. Upon direct irradiation ($\lambda \geq 300$nm) of the compound in acetonitrile or benzene, a *syn* head-to-tail C_4-cyclodimer is obtained ($\Phi = 0.07$) while an *anti* C_4-cyclodimer is obtained in the presence of triplet sensitizers such as benzophenone. Limettin undergoes C_4-cycloaddition reaction via the singlet exciplex when irradiated with tetramethylene with quantum yield of 0.16. The fluorescence of limettin is quenched by tetramethylene with the k_q of 4×10^9 mole^{-1} sec^{-1}. Photolysis of limettin with thymine and thymidine yields a C_4-photocycloadduct through 2+2 cycloaddition between pyrone double bond of limettin and 5, 6-double bond of thymine and thymidine. The biological importance of the reaction is discussed in comparison with that of psoralens.

Introduction

Furocoumarins, a group of naturally occuring substances in plants of the families *Umbilliferae* and *Rutaceae*, are known to photoreact with pyrimidine and purine bases in DNA under irradiation with near UV light, exerting various biological actions such as erythema on human and guinea pig skin, lethal and mutagenic effects in bacteria, inactivation of DNA viruses, and inhibition of the tumor-transmitting capacity of various tumor cells.[1] Derivatives of furocoumarin have been used for many years in the experiments of skin photosensitization, in the treatment of vitiligo and psoriasis and

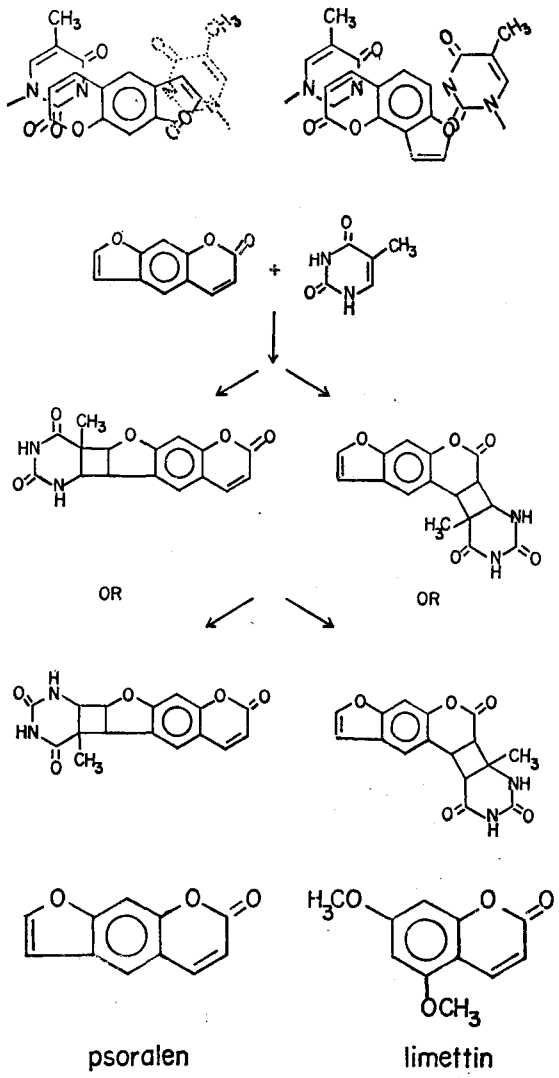


Fig. 1. Projection of the psoralen and angelicin molecule intercalated between two base pairs in DNA

recently, as molecular probes for chromatin structure[2,3,4], hairpin structure in viral DNA and in Drosophila ribosomal RNA[5,6] satellite DNA structure[7], and viral DNA-RNA hybrid structure[8].

The formation of interstrand cross-linking through C_4-cycloaddition of 3,4-and 4′, 5′-double bonds to the 5, 6-double bond of the pyrimidine bases, especially thymine, in DNA has been correlated with biological effects of photoexcited furocoumarins[9-13] without firm chemical evidences. The pyrone 3,4-and furan 4′,5′-double bonds are both necessary for the photosensitizing activity of furocoumarins but limettin without the furan ring in the molecule, intercalates strongly with DNA and photobinds covalently to DNA bases causing the same biological activities as furocoumarins, unlike most other coumarins[14].

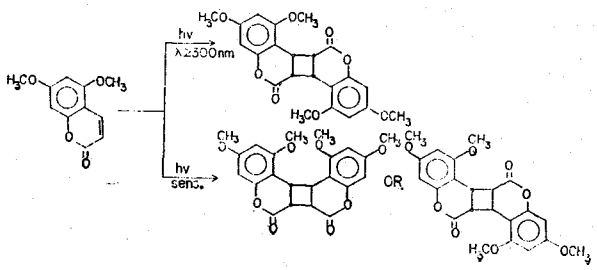
The isolation and characterization of furocoumarin-pyrimidine base photoadducts are absolute necessity for elucidation of molecular mechanism of the biological activities of furocoumarins but have been unsuccessful because of the complexities and lack of the quantities of the photoproducts of these compounds. The lack of bifunctionality in limettin warrants a lesser number of photoproducts upon irradiation with thymine compared to those of furocoumarins. The photocycloaddition reaction of limettin is therefore studied as a model for the psoralen-DNA C_4-cycloaddition reaction.

Photocyclodimerization of limettin Upon direct irradiation ($\lambda \geq 300$nm) of limettin in acetonitrile or benzene, a white crystalline precipitate was obtained. The structure of the compound was elucidated by mass, infrared, UV, and NMR spectra. Mass spectra showed the parent peak of m/e 412 corresponding to a limettin dimer and the base peak of m/e 206 (limettin). The infrared spectra showed $\upsilon_{C=O}$ at 1,760 cm^{-1} ($\upsilon_{C=O}$ of limettin: 1,710cm^{-1}) and UV absorption maximum shifted to 285 nm from 325 nm of limettin indicating the C_4-photocycloaddition of 3,4-double bonds. The nmr study showed no pyrone double bond proton absorptions(δ 7.90, 6.09) supporting the results of infrared and UV spectra. The comparative nmr study showed the configuration of the cyclodimer to be *syn* head-to-tail.

In the presence of triplet sensitizers such as benzophenone, a different crystalline C_4-photocyclodimer was obtained with the quantum yield of 0.08. The structure of the dimer was elucidated to be an *anti* C_4-cyclodimer by IR, UV, NMR and mass spectrometry.

The quantum efficiency for the synhead-to-tail dimer formation is 0.068, showing that the dimerization of limettin is markedly enhanced by the substitution of two methoxy groups at 5 and 7 positions. Azulene does hot quench the formation of the dimer in acetonitrile. Therefore the reaction is thought to proceed via limettin singlet state exclusively in direct irradiation. The luminescence data ($\Phi_F=0.65$, $\tau_F=7.2$ ns at 298 K: Φ_P/Φ_F 0.05 at 77K) support the mechanism. Additional evidence is that direct and sensitized excitations of limettin gave different dimers.

When the *syn* or anti dimer of limettin in acetonitrille (0.1nm) were irradiated with the light ($\lambda \geq 300$nm), no limettin was observed showing the new λ_{max} at 292nm. Therefore, the



lack of photosplitting C_4-cycloadducts should not be taken as a sufficient evidence against the C_4-photocycloaddition reactions[15].

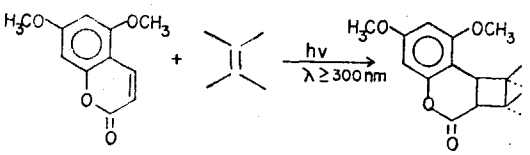
Photocycloaddition of Limettin to Tetramethylene: The photocycloaddition reaction of limettin to tetramethylene (TME) is studied as a model for the limettin-thymine C_4-cycloaddition reaction.

A solution of limettin (2.42×10^{-3} mole) and TME (0.0337 mole) in 500 ml methanol was irradiated through a Pyrex glass filter with 350 nm light in a Rayonet reactor for 9h. A TLC analysis(silica gel, cyclohexane-acetone 45 : 55 v/v) shows limettin-TME 1 : 1 adduct at R_f 0.95, limettin at 0.09, limettin cyclodimer at 0.85 along with several other minor products. The limettin-TME 1 : 1 adduct, m.p. 89.5°C, formed with a quantum yield of 0.16 is isolated by column chromatography (Wako gel C-200; cyclohexane-acetone solvent 45 : 55v/v).

Elemental analysis data(C, 70.3 and H, 7.76) agreed with the molecular formula $C_{17}H_{22}O_4$ (Limettin+TME). Calc. C, 70.3 and H, 7.64. The infrared spectrum shows a large blue shift of the strong carbonyl stretching band from 1,710 cm^{-1} in limettin to 1,755 cm^{-1} and a geminal methyl doublet at 1,380 cm^{-1}. The UV absorption spectrum shows λ_{max} at 283 nm but no limettin absorption maximum at 325 nm. These observations are consistent with the C_4-photocycloaddition of the pyrone double bond in limettin to TME, as the conjugated enone chromophore is deconjugated leaving only a simple substituted benzene chromophore to absorb light as shown in limettin dimerization, The NMR spectra shows four methyl groups at 1.27, 1.22, 1.02, and 0.75 (singlets) which are exactly the same as those of the photosensitized C_4-cycloaddition product of coumarin to TME. Two aromatic protons (δ 6.2, s), six methoxy protons (δ 3.78, s) and two cyclobutyl protons in an AB pattern at δ 3.47 and 3.18 with a coupling constant of J=10 Hz unequivocally prove the structure of the product to be C_4-cycloadduct of limettin to TME.

When the photoadduct in methanol is irradiated with 254nm light, photosplitting of the limettin-TME adduct resulted, further substantiating the cyclobutane structure of the product.

To elucidate the mechanism of this reaction, the fluoresence quenching of limettin is studied. Limettin has strong fluorescence and very weak phosphorescence(Φ_F, 0.65; τ_F, 7.2 ns at 298K; Φ_P/Φ_F, 0.05 at 77K) unlike other coumarins and furocoumarins and its photochemical reactivity is expected to be different from that of other coumarins. Coumarin itself, for example, reacts with TME photochemically only in the presense of sensitizers such as benzophenone to form a C_4-cycloadduct. TME quenches the fluorescence of limettin very efficiently with the quenching constant, k_q, of $4.0\times10^9 l$ $mole^{-1}$ sec^{-1} obtained from the linear Stern-Volmer plot. It is, therefore, very likely that an exciplex is formed between the limettin excited singlet state and the groud state of TME prior to cycloaddition and no triplet sensitizer is necessary for limettin to add to TME photochemically[16].



Photocycloaddition of limettin to thymine: C_4-photocycloaddition of limettin to thymine ($\lambda\geqslant300$nm) was studied in dioxane-water (1 : 10) solution, in aqueous frozen state, and in solid film state varying the concentration ratio of limettin to thymine. The rate of limettin disappearance was enhanced as the concentration of thymine increased indicating

the cross reaction between limettin and thymine in addition to limettin C_4-photocyclodimerization reaction.

The photolysis products of limettin and thymine were diagnosed by TLC and two cross addition products were detected. The 1 cm thick aqueous frozen solution (Limettin: Thy= 1 : 10) yielded the photoaddition products in the largest quantity. One major product and the other in trace quantity were detected. The major product was isolated by silca gel column chromatography and recrystallization from water. When 400mg of limettin and 2.5g of thymine were irradiated in frozen aqueous solution, 20 mg (about 3% yield based on limettin) of the major product was obtained after the column chromatography and recrystallization. Over 350 mg of the photoadduct was collected and used for the characterization.

It has a sharp melting point 259.5°C measured by DSC. The elemental analysis data are consistent with the molecular formular of a 1 : 1 adduct of limettin and thymine, $C_{16}H_{16}$-N_2O_6, as shown below.

Calcd. for $C_{16}H_{16}N_2O_6$: C, 57.83; H, 4.82; N, 8.43; O, 28.92.
Found: C, 57.98; H, 4.73; N, 8.11; O, 29.18.

The mass spectra of the photoadduct was determined by electron impact (EI) and chemical ionization (CI) methods. A small molecular ion peak corresponding to a 1 : 1 limettin-thymine adduct was observed at m/e 332 from the EI method and a quasi-molecular ion at m/e 333 from the methane gas CI method. When ammonia is used in the CI method, a strong peak was observed at m/e 350 which corresponds to the moecular ion (332) plus ammonium ion [18] and at m/e 367 which is formed by the addition of another ammonia moleucle to 350 species. The fragmentation patterns show the base of m/e 207 (limettin) and are very similar to those of a limettin and thymine mixture, indicating efficient limettin and thymine formation by splitting of the photoadduct. This suggests that the photoadduct is a C_4-photocycloaddition product of limettin and thymine formed through 2+2 addition of the 3,4-double bond of limettin to the 5,6-double bond of thymine. The low intensity of the molecular ion peak, only 0.2% of the base peak intensity, strongly supports this proposition since the molecular ions of cyclobutane dimers of limettin and pyrimidine bases are known to fragment readily into ions of lower mass in EI method as well as decomposing thermally prior to ionization.

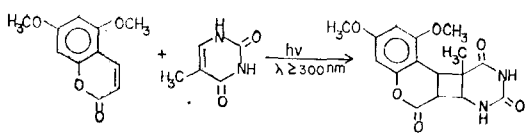
The UV absorption spectrum of the photoadduct is nearly superimposable on that of the limettin dimer and shows the loss of conjugation in limettin by the blue shift of λ_{max} from 324nm to 285nm. The thymine absorption band at 260nm disappears as well as suggesting the 2+2 cycloaddition of the limettin 3,4-double bond to the 5,6-double bond of thymine.

The infrared spectra show the strong carbonyl stretching band of limettin at 1,710cm^{-1} shifted to 1,761cm^{-1} which is identical with that of the limettin dimer and similar to that of C_4-cycloadduct of limettin to tetramethylethylene. This along with the UV spectra strongly support the hypothesis that the pyrone double bond is lost on photoreaction. The other $\lambda_{C=O}$ band at 1,718cm^{-1} is very close to that of thymine C_4-cyclodimer at 1,715cm^{-1} indicating the loss of the 5,6-double bond of thymine in the photoproduct. A characteristic cyclobutane ring vibrational band at 860cm^{-1} which is not observed in limettin or thymine spectra is apparent. The remainder of the spectra is similar to that of the sum of C_4-cyclodimers of thymine and limettin further suggesting the

C_4-cycloaddition of limettin to thymine.

The proton nuclear magnetic resonance spectra of the photoadduct taken in pyridine-d_5 show neither pyrone double bond protons at δ 6.09 and 7.90 nor the thymine olefinic proton at δ 7.24, again indicating the loss of the 3,4-double bond of limettin and the 5,6-double bond of thymine through 2+2 cycloaddition on photolysis. New signals at δ 3.93 (d, 2H) and δ 4.57 (q, 1H) are observed in a spectral region typical for cyclobutane protons. The remainder of the spectra is consistent with the cyclobutane adduct structures of limettin-thymine.

The C_4-photocycloaddition and its reversal photosplitting are one of the characteristic properties of cyclobutane pyrimidine dimers, limettin dimers, and limettin-TME C_4-photoadducts. The crystalline photoadduct of limettin to thymine is also very sensitive to 254nm light. When the aqueous solution of the photoadduct was irradiated with 254nm light for 2~5 minutes, limettin and thymine were obtained as detected by UV (λ_{max} 324nm) and TLC analyses indicating the product to be a C_4-cycloadduct of DMC and thymine. When tryptophan and chloranil were used as photosensitizers, the photoadduct was also split into DMC and thymine. Thus the spectral analyses, photosplitting of the product, and elemental analysis data conclusively indicate the photoproduct to be cyclobutane type cross addition product of limettin and thymine[17].



Photoaddition of limettin to thymidine: A mixture of limettin and thymidine, a nucleoside, was irradiated in aqueous solution, aqueous frozen state, and in solid film state. The same number of cross photoaddition products was detected for each case on TLC plates.

Photoproducts of limettin and thymidine were detced by TLC and several cross products were observed. The film state (Limettin: Thymidine =1.:5 mole ratio) gave best results yielding one major product (chemical yield 9%) and the others in trace quantities.

The major product was isolated by Sephadex G-10 column chromatography, followed by silica gel column chromatography and recrystallized from ethyl acetate.

The mass spectrum of photoproduct was determined by electron impact method. It is well known that the molecular ions of cyclobutanes such as cycloadduct of limettin and thymine readily fragment into ions of lower masses as well as decomposing thermally prior to ionization. A small molecular ion peak corresponding to 1 : 1 limettin-thymidine adduct was observed at m/e 443 and a peak of m/e 332 (1 : 1 limettin-thymine) was also observed. The fragmentation patterns are very similar to those of DMC and thymidine mixture, indicating an efficient regeneration of limettin and thymidine by splitting of the photoadduct. These results suggest that the photoadduct is a C_4-photocycloaddition products of limettin and thymidine through 2+2 addition of 3,4-double bond of limettin to 5,6-double bond of thymidine.

The elemental analysis determined by micro method was found to be in good agreement with the monohydrated molecular formula of a 1 : 1 cycloaddition product of limettin and thymidine, as shown below.

Calcd. for $C_{21}H_{24}N_2O_9$: C, 57.25; H, 5.39; N, 6.25

Calcd. for $C_{21}H_{24}N_2O_9 \cdot H_2O$: C, 54.07; H, 5.57; N, 6.01

Found: C, 54.04; H, 5.51; N, 6.01

The UV absorption spectrum of the photoadduct was nearly superimposable with that of limettin dimer and 1 : 1 limettin-thymine adduct; thus, the loss of conjugation in limettin-thymidine adduct shows blue shift of λ_{max} from 324nm to 285nm. The λ_{max} at 267nm of thymidine disappeared, suggesting the formation of an adduct between limettin and thymidine.

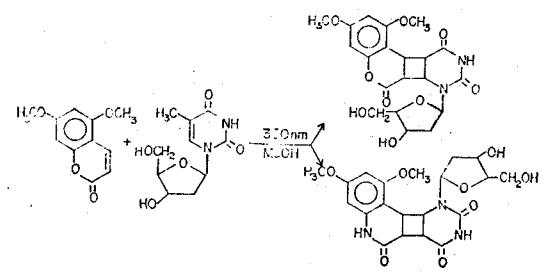
The infrared spectrum shows a shift of the carbonyl stretching band from 1,710cm^{-1} in limettin to 1,750cm^{-1} in photoproduct, which is similar to that of C_4-cycloadduct of limettin to tetramethylethylene and C_4-cycloadduct of limettin to thymine. The IR data thus clearly show the saturation of pyrone double bond on photoreaction. The spectrum also shows the shift of 1,660cm^{-1} band to 1,690cm^{-1}, indicating the loss of 5,6-double bond of thymidine in the photoproduct. Broad ν_{O-H} and ν_{N-H} stretching bands are apparent. A characteristic cyclobutane ring vibration at 830cm^{-1} which is absent in limettin or thymidine spectra is also distinct. The remainder of the spectrum is very similar to that of cycloaddition product of limettin to thymine.

The proton NMR spectrum of the photoadduct taken in pyridine-d_5 showed neither the pyrone double bond protons of limettin at δ 6.09 and δ 7.90 nor the thymidine olefinic proton at δ 8.10, again indicating the loss of the 3,4-double bond of thymidine through 2+2 photocycloaddition. The methyl proton signal of thymidine (δ, 1.89) shifted 0.21ppm upfield (δ, 1.68), and 1′-H triplet peak of thymidine (δ, 6.98) shifted 0.15ppm upfield (δ, 6.83) in the photoadduct. New signals at δ 3.95 (d, 2H) were observed in a spectral region typical for cyclobutane protons. Unfortunately, peak for one proton in the cyclobutane ring cannot be resolved probably because it is superimposed with hydroxyl and aliphatic protons in deoxyribose ring. The remainder of spectum is consistent with the cyclobutane limettin-thymidine adduct structure, and the spectrum is very similar to that of C_4-cycloadduct of limettin to thymine.

For the 2+2 photocycloaddition of limettin to thymidine, four different *cis*-fused cyclobutane geometrical isomers are theoretically possible; *syn* head-to-head, *syn* head-to-tail, antihead-to-tail. Furthermore eight different stereoisomers are possible because of the 1′-asymmetric carbon in thymidine.

From the vicinal coupling constants and chemical shifts of cyclobutane protons, the configuration of the photoadduct is thought to be *syn* head-to-tail, but more thorough investigation is necessary to establish the configuration of photoadduct definitely.

The C_4-photocycloadduct of limettin-thymidine in methanol undergoes reversible photosplitting, yielding limettin and thymidine on irradiation with 254nm UV light for 1 minute, as is the case with the photosplitting of P_{yr} ◇ limettin, limettin ◇ limettin, TME C_4-photoadducts, and limettin ◇ Thy. Limettin and thymidine regenerated were detected by UV(λ_{max} 324 nm) and TLC analysis, indicating the product to be a C_4-cycloadduct of limettin-thymidine.



Photosplitting of C_4-cyclodimers of limettin: The photosplitting of limettin C_4-cyclodimers with 254nm UV light and by triplet

sensitized excitation in methanol or acetonitrile is studied to see the repair mechenism of photomodified DNA by psoralens and limettin. The *syn* dimer is splitted into limettin through the singlet excited state while the *anti* dimer is splitted through the excited triplet state. The photolysis product, limettin, was detected by UV and TLC and quantitatively measured by monitoring the absorbance at 324nm (λ_{max} of limettin).

Conclusion

The results suggest that the limettin-DNA adduct is likely a C_4-cycloadduct of limettin to the thymine moiety of DNA as supported by analogy with CD data[18]. Thus C_4-cycloproducts strongly support the proposition that C_4-cycloadduct formation is the major cause of photobiological effects of limettin and psoralens and the photoadducts should be very useful in elucidating the possible cycloaddition products between DNA and limettin or psoralens and in the study of molecular mechanism of photosensitization reactions of these compounds.

Acknowledgements:

This investigation was supported by Grant Number 5 RO 1 CA 21729, awarded by the National Cancer Institute, DHEW, U.S.A.

References

1. Sunlight and Man, Edited by M.A. Pathak, L.C. Harber, M. Seiji, and A. Kukita, University of Tokyo Press, Tokyo, 1974
2. Hansen, C.V., C. K.J. Shen, and J.E. Hearst, *Science*, **193**, 62 (1976)
3. Wiesehahn, G.P., J.E. Hyde, and J.E. Hearst, *Biochemistry*, **16**, 925 (1977)
4. Cech, T and M.L. Pardue, *Cell*, **11**, 631 (1977)
5. Shen, C. K.J. and J.E. Hearst, *Proc. Natl. Acad. Sci.*, **73**, 2649 (1976)
6. Wellerzien, P.L., D.C. Youvan, and J.E. Hearst, *ibid.*, **75**, 1642 (1978)
7. Shen, C. K.J. and J.E. Hearst, *J. Mol. Biol.*, **112**, 495 (1977)
8. Shen, C. K.J., T.S. Hsieh, J.C. Wang, and J.E. Hearst, *ibid.*, **116**, 661 (1977)
9. Musajo, L., F. Bordin, and R. Bevilacqua, *Photochem. Photobiol.*, **6**, 927 (1967)
10. Musajo, L., F. Bordin, G. Caproale, S. Marciani, and G. Rigatti, *ibid.*, **6**, 711 (1967)
11. Musajo, L. and G. Rodighiero, *ibid.*, **11**, 27 (1974)
12. Krauch, C.H., D.M. Krämer, and A. Wacker, *ibid.*, **6**, 341 (1967)
13. Krauch, D. M. and M.A. Pathak, *ibid.*, **12**, 333 (1970)
14. Ou, C. N., P.S. Song, M.L. Harter, and I.C. Felkner, *ibid.*, **24**, 487 (1977)
15. Sang Chul Shim, Kwan Yong Choi, and Pill-Soon Song, *Photochem. Photobiol.*, **27**, 25 (1978)
16. Sang Chul Shim and Dae Yoon Chi, *Chem. Letters*, No. 11, 1129 (1978)
17. Sang Chul Shim and Kyu Ho Chae, *Photochem. Photobiol.*, **30**, 349 (1979)
18. C. N. Ou, Ph.D. Dissertation, Texas Tech University, Lubbock, Texas (1977)