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INVITED REVIEW

PHOTOCHEMISTRY AND PHOTOBIOLOGY OF PSORALENS

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INTRODUCTION

Psoralens are planar tricyclic furocoumarins present in numerous plants and fungi found throughout the world.[1] Naturally occurring and synthesized psoralen derivatives(see Figure 1) are photosensitizers of UVA especially from 320 nm to 400 nm, a range at which cellular nucleic acids and proteins are weakly absorbing if any at all. Because of their skin-photosensitizing properties, these compounds have been used in the photochemotherapy of psoriasis and vitiligo.[2,3] However, undesirable side effects such as carcinoma development in hairless mice as well as possible liver damage from the use of 8-methoxypsoralen(8-MOP) have been reported.[4] The other photobiological effects include inactivation of DNA viruses, killing and mutagenesis of bacteria, inhibition of tumor transmitting capacity of various cells, and hyperpigmentation on human and guinea pig skin .[5,6]

PUVA(psoralen+UVA) photochemotherapy is in fact thousands of years old, having been used in Egypt and India since B.C. 1200-2000. Photochemotherapy for a common disfiguring disease, vitiligo, was practiced in the ancient world by physicians and herbalists who used boiled extracts of the fruits of certain umbelliferous plants, *e.g. Ammi majus Linnaeus* in Egypt or the leguminous plants, *Psoralea corylifolia L.* in India.[7]

It was first described by Kuske in 1938[8] that photosensitization of skin by plants was related to the presence of psoralen. He identified natural psoralens in plants as photosensitizers and isolated bergapten(5-methoxypsoralen) from the oil of bergamot. The scientific interest in photosensitizing psoralens, however, has grown considerably after the introduction into clinics of the psoralen photochemotherapy for the treatment of psoriasis and of other skin diseases by Parrish *et al.*[2] At present, 8-MOP, 5-MOP and 4,5',8-trimethylpsoralen(TMP) are employed in the PUVA photochemotherapy. The discovery of PUVA photochemotherapy stimulated new studies directed towards a better knowledge of the photosensitization mechanisms, of the action of psoralens and towards the other effective applications of psoralens.

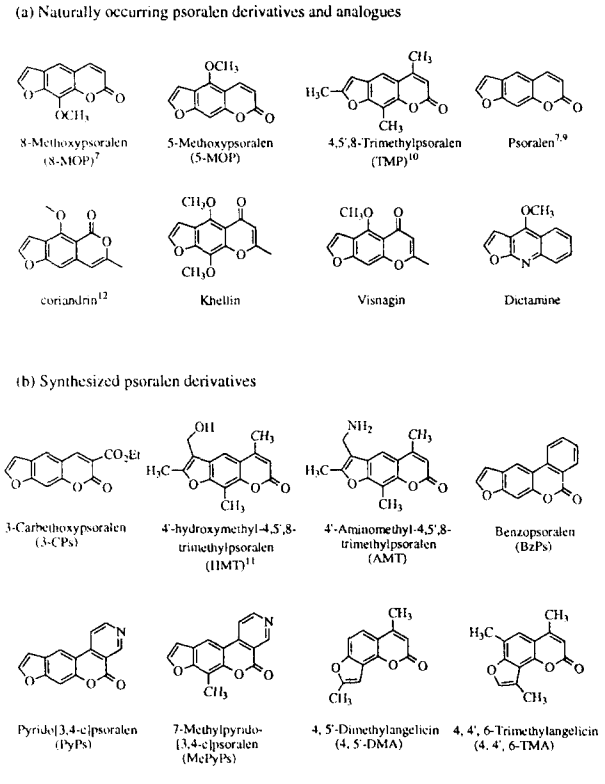

Figure 1. Molecular structures of naturally occurring and synthesized psoralen derivatives

Recent progress towards an understanding of psoralen photosensitization mechanisms comes from development of new psoralens, more detailed elucidation of the photoreaction of new psoralens with nucleic acids including wavelength specificity and reirradiation protocol, detection and characterization of DNA adducts formed *in vitro* [13-15] and

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vivo,[16] analysis of reactivity with biomolecules other than nucleic acids(*e.g.* lipids and proteins) and involvement of oxygen mediated reactions.[17] More recently the successful treatment of the pruritus of human immunodeficiency virus(HIV-1) infection and acquired immunodeficiency syndrome(AIDS) with PUVA have been reported.[18]

In addition, encouraging results have been obtained for the treatment of cutaneous T-cell lymphoma and other T-cell mediated diseases such as pemphigus vulgaris, psoriatic arthropathy and chronic lymphocytic leukemia with extracorporeal irradiation of 8-MOP containing blood.[19]

Besides medicinal applications, psoralens have been proven to be useful tools as molecular probes in studies dealing with chromatin structure, secondary structure in viral DNA repair mechanisms and viral DNA-RNA hybrid structure.[20]

PHOTOPHYSICAL PROPERTIES

The intrinsic photoreactivity of psoralens is determined by the electronic structure of the lowest excited states(S_1 and T_1).[21,22] The UVA bands of all psoralen derivatives arise from their pyrone moiety.[5,7,23] The hydrogenation of the pyrone 3,4-double bond abolishes the long wavelength UV band of psoralen. In psoralen, the $^1(n,\pi^*)$ state provided by carbonyl

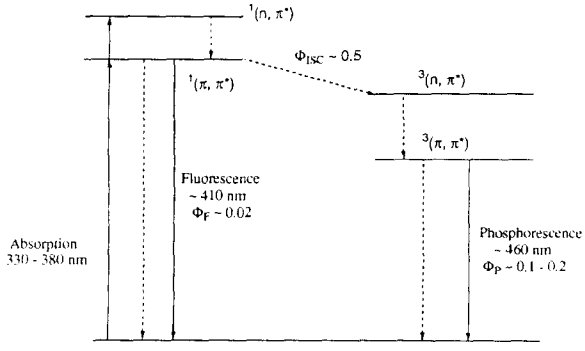


Figure 2. The energy levels for low-lying excited states of psoralens (psoralen as a typical case)

group lies just above the lowest $^1(\pi,\pi^*)$ state. Figure 2 shows approximate energy level of psoralens.[24]

The short lifetime of the $^1(\pi,\pi^*)$ state of some psoralen derivatives can be explained in terms of the closeness of $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states(see Figure 3).[25,26] Electron-donating substituents, such as methyl (as in TMP) and methoxy (as in 8-MOP)groups, will raise the $^1(n,\pi^*)$ state and thus enhance the photoreaction by way of the singlet state due to a weakening the vibronic interaction between the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states. Electron-withdrawing substituents such as carbethoxy group(as in 3-CPs) tend to lower the $^1(n,\pi^*)$ state and, therefore, the photoreactivity of the $^1(\pi,\pi^*)$ state of 3-carbethoxypsoralen may be significantly lower than that of the $^1(\pi,\pi^*)$ state in other psoralens. Closeness of the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states causes vibronic(vibrational and electronic) interaction between two states and the shortening of the lifetime of the lowest excited states and thus the excited $^1(\pi,\pi^*)$ state becomes kinetically and electronically less reactive. Another consequence of close lying $^1(n,\pi^*)$ state is the enhancement of intersystem crossing to the $^3(\pi,\pi^*)$ state through vibronic spin-orbit coupling and through the intervening $^3(n,\pi^*)$ state, which is positioned between the $^1(\pi,\pi^*)$ and $^3(\pi,\pi^*)$states.

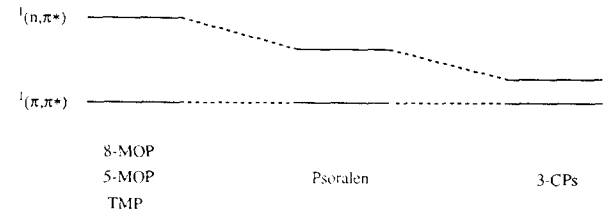


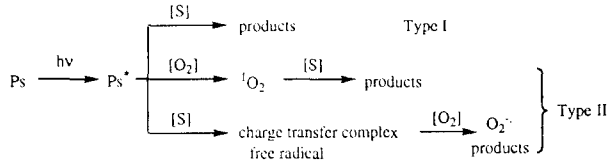
Figure 3. Approximate energy level of excitied singlet state of psoralen and its derivatives

The cycloaddition reactivity of either the $^1(\pi,\pi^*)$ or $^3(\pi,\pi^*)$ toward pyrimidine is determined by kinetic(i.e. lifetimes), steric and electronic factors. The electronic factors consist of the degree of local excitation and the electron density at the 3,4 and 4',5'double bond in the excited state. They exhibited the significant differences. The 3,4 double bond of pyrone is locally excited in the triplet state, not observed in the 4',5' double bond. Theoretical calculations of the electron density in these excited states of psoralen show that the 3,4 double bond has more charge tranfer character than the 4',5'double bond.[27] Marko *et al.* have pointed out that in all the furocoumarins(psoralens) the 3,4 - pyrone double bond protons were always the most polarized.[28] Based on these results, it has been suggested that the triplet state photoreactivity resides with the pyrone moiety rather than with the furyl group.

The photophysical properties, however, differ appreciably from one compound to another in this wavelength region (see Table 1). The lowest singlet and triplet states of psoralens have the (π,π^*) electronic configuration and properties of the excited states are very sensitive to the change of their environments, *i.e.*, solvent polarity. Solvent effects of psoralen derivatives are explained by redistribution of charge at both the 3,4 and 4',5' double bond and the proximity effect due to (n,π^*) as described above.

PHOTOCHEMICAL PROPERTIES

The excited states of psoralens undergo various photochemical reactions with another ground psoralen molocules, solvent and biomolecules *via* type I and type II mechanisms(Scheme 1). Type I mechanism involves the generation of free radicals and the direct photobinding to the substrate.[29] Biologically important cycloadditions include those occurring between psoralens and the pyrimidine bases of nucleic acids[30,31] or unsaturated fatty acids.[32,33,34] These reactions require no molecular oxygen and play the major role in the induction of lethal and mutagenic effects in micro-organisms, viruses and mammalian cells.[35,36,37] Also the photochemical events connected with the generation of free radicals appertain to this mechanism.



Ps: psoralen; Ps*: excited psoralen; [S]: substrate

Scheme 1. Photoreaction mechanism of psoralens

Type II mechanism, on the other hand, involves an indirect photochemical reaction; for example, the triplet-excited psoralens transfer their energies to molecular (triplet) oxygen leading to the formation of singlet molecular oxygen. This reactive species of oxygen has lifetime of about 2μs in water and can therefore react with important biomolecules. In another type II reaction the triplet-excited psoralens reduce molecular oxygen to the reactive superoxide radical anion,[38,39,40] a precursor of hydrogen peroxide.

For psoralens it has been clearly shown that both type I and II mechanisms are involved in the photosensitization processes leading to many photobiological consequences.

Table 1. Absorption(λ_{max},ε), Fluorescence(Φ_f)and Triplet-state yield(Φ_t) of Psoralens[a]

compound	λ_{max},(ε)	λ^f_{max}	Φ_f	Φ_t
8-methoxypsoralen	246(21,000) 303(12,014)	508	0.0015	0.06
	299	460	0.0020	0.04
5-methoxypsoralen	312(14,200)	460		<0.01
	355	427	0.019	0.1
4,5',8-trimethylpsoralen	250(15,000) 298(7,950) 335	430	0.056	0.09[b]
	337	416	0.044	
psoralen	330	409	0.019	0.13
	244[c] 295 335	444	0.01	0.06
angelicin	246 300(9,530)		0.33	
3-carbethoxypsoralen	247 318(10,900) 365	460	0.02	0.35

[a] measured in water, [b] measured in methanol, [c] measured in water/ethanol, [d] measured in ethanol

PHOTOBIOLOGY AND APPLICATIONS

PHOTOREACTION WITH BIOMOLECULES

The mechanisms of the photobiological activity of psoralens have not yet been fully understood. However, they have been related to photomodifications and/or photobinding to the cellular macromolecules,[41] such as nucleic acids,[42] proteins[43] and membrane lipids[44] (see Scheme 2).

Ps + UVA
↓
Reactions with biomolecules

Without O_2 participation: Addition to DNA. Addition to fatty acids. Covalent binding to proteins.
Photodynamic reactions (Ps+UVA+O_2) : Covalent binding to proteins. Oxidation of amino acids. Cross-linking of proteins. Inactivation of enzymes. Destruction of cytochrome P-450. Oxidation of SH compounds. Oxidation of lipids. Oxidation of tocopherol. Oxidation of DOPA. Oxidation of DNA.

↓
Cellular effects

Inhibition of synthesis of nucleic acids, proteins and cell growth. Lethal effect. Mutations. Induction of sister chromatid exchange. Simian virus induction from a mammalian cell. Inactivation of ribosomes. Impairment of oxidative phosphorylation in mitocondria. Increase in membrane permeability. Destruction of receptors of epidermal growth factor.

↓
Effects on the organism

Therapeutic effects: Vitiligo. Psoriasis. Alopecia areata. Mycosis fungoides. Dyshidrotic eczema. Sterilization of PCR carry-over. Sterilization of human blood fraction for transfusion. Other effects: Erythema. Oedema. Hyperpigmentation. Skin cancer. Cataracta. Suppression of melatonin secretion. Prolongation of skin graft survival in mice.

Scheme 2. Photoreaction of psoralens with biomolecules. from A. Ya. Potapenko (1991).

Psoralen monoadducts and diadducts with nucleic acids. The best known photochemical reactions of psoralens are those with nucleic acids, especially DNA.[45] It appears that the genotoxic effects as well as the therapeutically important antiproliferative effects are due mainly to their capacity to induce photoconjugation to DNA. The modification of DNA by psoralens is thought to be a two-step process[46,47]: (a) formation of a molecular complex in the ground state; (b) photoconjugation of the complexed psoralen to pyrimidine bases of DNA(see Figure 4).

Psoralens show an evident affinity for DNA in the dark; they form molecular complexes with the macromolecules by undergoing intercalation between two base pairs of the DNA. This reaction is reversible, and the rates of the forward and backward reactions are affected by the solubility of both nucleic acid and psoralen in the solution in which the reaction takes place. The position assumed to be intercalated by psoralens stongly favors their cycloaddition with the pyrimidine bases of DNA. In this connection it is interesting to observe the different possibilities of cycloaddition between psoralens and angelicins. While for intercalated linear furocoumarins(psoralen) both photoreactive sites can be aligned with two 5,6-double bonds of two pyrimidine bases, disposed one above and one below the planar chromophore of the furocoumarin (psoralen), for intercalated angelicins on the other hand as a consequence of their angular structure, only one of their photoreactive site can be aligned with a pyrimidine base of DNA. Psoralen can therefore engage both their photoreactive sites joining covalently with two pyrimidines each appertaining to a complementary strand of the macromolecule forming 1:2 furocoumarin-thymine diadduct and giving rise to interstrand cross-linkages in the macromolecule. Angelicins, on the other hand, can engage only one photoreactive site giving rise to 1:1 furocoumarin-pyrimidine monoadduct. However, recently Bordin *et al.* reported that 4,6,4'-trimethylangelicin, a well-known effective photosensitizer described as a pure monofunctional reactant with DNA, can induce interstrand cross-links in mammalian cell DNA *in vivo* (about 15% relative to 8-methoxypsoralen).[48]

In other words the formation of the preliminary intercalated complex between furocoumarins and DNA is an important step which markedly affects the successive covalent photobinding to the macromolecule.

Psoralens have two different photoreactive sites, 3,4-and 4',5'-double bonds; therefore, two types of monoadducts, that is 3,4- and 4',5'-monoadducts, can

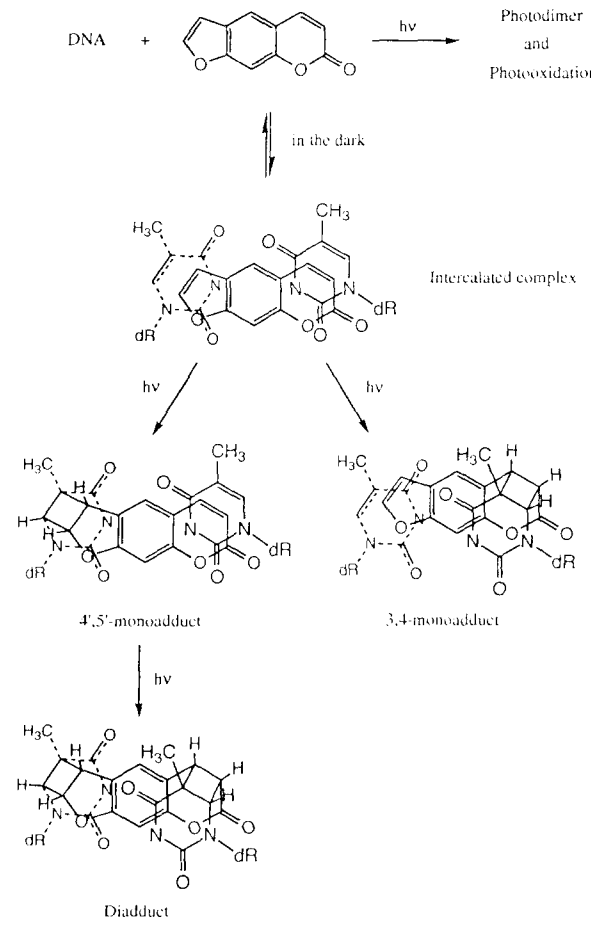


Figure 4. The overall sequence of the photochemical reaction between psoralen and DNA

be formed by the C_4-photocycloaddition with 5,6-double bond of a pyrimidine base of DNA.[45] In particular, 4',5'-monoadducts once formed can absorb a second UVA photon and photoreact further engaging their 3,4-double bond,[49] *i.e.*, a second cycloaddition with a pyrimidine of the complementary strand of the macromolecule can occur forming interstrand cross-linkages in DNA. 3,4-Photoadducts of psoralens do not form cross-linkages on further irradiation with 365 nm UV light because they do not absorb this light. The *cis-syn* structure shown by both mono- and diadducts isolated from the irradiated mixture of various psoralens and DNA also supports the importance of preliminary complex in the ground state formed between furocoumarin and the DNA. The formation of a bifunctional adduct with the pyrimidine base of the opposite strand leads to an inhibition of DNA synthesis and cell division, and it is generally assumed, although not definitely proven, that cross-linking represents the mechanism

of the therapeutic effect in psoriasis.

In the formation of psoralen-DNA photoadducts, the relative importance of the singlet and triplet states of psoralens is not well established. From the theoretical studies,[50] it has been proposed that the psoralen triplet state should be the reactive precursor to monoadduct formation and this hypothesis was supported by both quenching studies in steady state photolysis and laser flash photolysis experiments[51,52,53] as well as by studies on the cross-link formation between DNA and 4'-aminomethyl-4,5', 8-trimethyl-psoralen(AMP).

However, since no evidence for triplet formation of the DNA-complexed psoralens was obtained and their singlet excited states were quenched by intercalation into DNA, singlet excited state mechanism has been suggested and probably better accepted for psoralen-DNA monoadduct formation.[54,55,56]

Recently, an evidence for the formation of triplet psoralen in DNA-intercalated complex is reported. The 365 nm irradiation of pyridopsoralen(PyPs)-thymidine and of PyPs-DNA aqueous solutions was found to lead to the formation of cyclobutane thymidine dimers, in addition to 4',5'-monoadducts. The thymine dimer formation photosensitized by pyridopsoralens proceeds by the way of a triplet energy transfer from pyridopsoralen to thymine because the triplet energy increases on pyridine fusion and becomes closer to thymine triplet energy and consequently thymine dimer production increases significantly.

Although excited psoralens mainly react with the pyrimidine bases of DNA and RNA, they are able to form adducts with purine nucleosides under certain conditions. In contrast to psoralen-thymidine photoadducts, the photobinding of 4,5',8-trimethyl psoralen (TMP) proceeds through covalent bond formation between carbon C(4) of pyrone ring and ribose 1'-, 5'- or 4'-carbons in a dry film.[57,58] Similar photoadducts are obtained in the photoreaction of 8-MOP with 2'-deoxyadenosine and of 5,7-dimethoxy-coumarin (DMC) with adenosine.[59,60]

Photoreaction with proteins and membrane lipids. For a number of proteins it recently has been shown that covalent bonds with psoralens can be formed on irradiation. The covalent photoaddition of psoralens to proteins is reported in solution,[61-65] in the erythrocyte membranes and in skin cell. The biological implications are not firmly established yet. It is conceivable that photooxidation of amino acid residues *via* type II mechanism is responsible for inactivation of enzymes while the covalent photoconjugation does not seem to play a role in this connection. Type II reaction also results in the cross-linking of enzyme subunits of oligomeric proteins such as glutamate dehydrogenase, catalase and alcohol dehydrogenase,[62] erythrocyte ghost proteins[66] and alpha-crystalline lens proteins.[67]

There is no correlation between PUVA-induced polymerization of the subunits of the oligomeric proteins and their inactivation; thus PUVA inactivation of glutamate dehyrogenase and catalase proceeds three times slower than polymerization. On the contrary, PUVA inactivation of alcohol dehydrogenase occurs at a faster rate than its polymerization. Moreover, there is no correlation between the number of psoralen molecules photoadded to protein and the amount of destroyed amino acid residues .

In the presence of molecular oxygen, psoralens sensitize the photooxidation of unsaturated lipids.[68,69,70] Among lipoperoxidation products of unsaturated fatty acids, malondialdehyde, other aldehydes, ketones and hydroperoxides have been identified.[69] The oxidative photoreactions of lipids occur by two mechanisms, *i.e.*, by UVA irradiation in the presence of psoralens, or by the addition of the previously photo-oxidized psoralens to aqueous suspension of liposomes. The lipid peroxidation may rapidly lead to degradation of the cell membrane and cause erythema, inflammation and skin oedema.[71,72] The photoexcited psoralens also photoreact with the olefinic double bond of unsaturated fatty acids by oxygen-independent photoaddition, forming cyclobutane adducts.[73-78] Addition is observed to occur at the 3,4-bond (pyrone double bond) of psoralens. The methylene group deriving from position 9 (or 10) of OAME (oleic acid methyl ester) and 10-H (9-H) of OAME are opposite side of the cyclobutane ring, *i.e.*, *trans* configuration, while 3-CH_3 and the 4-H of TMP are on the same face, *cis* configuration, in all TMP-OAME photoadducts. The *trans* fatty acid formed by *cis-trans* photoisomerization may be an intermediate for *trans-cis* photoadducts.[79]

On irradiating arachidonic acid in the presence of TMP, *Kittler* and *Lober* found no product of fatty acid oxidation. This negative result is due to the use of commercial preparations of arachidonic acid which usually contain considerable amounts of the oxidation products. In the experiments the psoralen-sensitized photooxidation occured only with phospholipids isolated immediately prior to treatment in which the content of the oxidation products is very low. Under these conditions, psoralens can photoinitiate the oxidation of unsaturated fatty acids and sensitize the destruction of the oxidation products; for linolenic acid, the higher the linolenate peroxide concentration, the greater was the number of free radicals produced.

PHOTOBIOLOGICAL APPLICATIONS

The psoralen derivatives isolated from over 30 plants or synthesized have been applied for determining the structure of both DNA and RNA in viral, bacterial and mammalian cell. The biological activity of psoralens has primarily been connected with photoreactivity towards DNA, particularly the ability of psoralens to form covalent linkages. This ability of psoralens has been used to form covalent hybrids for locating particular sequence and also for site-specific crosslink. Hanson *et al.* first applied psoralens as probes for chromatin structure based upon the results of an electron microscopic analysis of denatured DNA isolated from photoreaction mixture of TMP and *Drosophila embryo nuclei* in 1976.[80]

The site-specific crosslink of psoralen derivatives will allow the mapping of DNA and RNA secondary structure. For specific application such as site-specific crosslinking of DNA and protein-nucleic acid crosslinking, synthesized psoralen derivatives such as HMT(4'-hydroxymethyl-4,5',8-trimethyl psoralen) and isopsoralen derivatives were applied. They were used to study *in vivo* and *in vitro* RNA structure and recently led to a new distance geometry model for the 16S ribosomal RNA[81]. More recently the advent of nucleic acid hybridization method has revolutionized both the detection of infectious desease pathogons and the identification of genetic variation associated with human disease.

Sancar *et al.* reported enzyme systems which recognize and act on psoralen-DNA photoadducts by the excision-repair mechanism.[82] The exact size of the oligomers excised by the enzyme complex is different for the three psoralen adducts. Psoralens react with thymidine in DNA and uridine in RNA and to a lesser extent with cytosine in DNA and RNA, to form first monoadducts which are linked to only one strand of the helix. The monoadducts can be converted into diadducts if there is another adjacent pyrimidine base available for the cross-link.

Photoreaction of psoralens with thymine in DNA is detrimental to mechanism of a DNA to survive and replicate. In survival and replication, transcription is an important process. Transcription consists of three steps which are initiation, elongation and termination. Between psoralens and double-stranded nucleic acids it has been possible to construct a double-stranded DNA fragment containing an *E. coli* RNA polymerase promoter and a psoralen diadduct at a specific site.[83,84] 4'-Hydroxymethyl-4,5',8-trimethylpsoralen(HMP) was photochemically attached to two adjacent thymidine bases on opposite strands as a dT-HMT-dT. It has been demonstrated that an elongation complex was formed on a DNA template bearing a site-specific psoralen cross-link (+36T on the top strand and +37T on the bottom strand)when the process of T7 RNA polymerase was arrested by the crosslink. Transcription of the cross-linked DNA was blocked by the bifunctional psoralens (photodiadducts).

These psoralens have recently been used in sterilization to avoid polymerase chain reaction (PCR) carry-over and sterilization of human blood fractions for transfusion.[85] More recently post-PCR sterilization has been applied to diagonistic assay for human immunodeficiency virus(HIV-1) with the 115-mer HIV-1 amplicon. This amplicon is widely used for the detection of HIV-1. The sequence is relatively short, which provided a challenging test for the sterilization technique since sterilization becomes increasingly difficult with amplicon of decreasing size. By providing sufficient target molecules for analysis by nucleic acid hybridization method, the polymerase chain reaction has become a key component in development of hybridization-based clinical diagnostic tests.

Isaacs *et al.* have developed and characterized an effective procedure for post-PCR sterilization which satisfies both the inactivation and hybridization requirements of a practical sterilization procedure with isopsoralen derivatives. They have found that isopsoralen derivatives(4'-aminomethyl-4,5'-dimethylisopsoralen,6-aminomethyl-4,5'-dimethylisopsoralen) have the properties which have minimal inhibitory effects on PCR but provide sufficient number of covalent adducts which are effective polymerase blocks .

Ceska *et al.* have characterized[12] and isolated coriandrin which is a furoisocoumarin from *coriandrum sativum L.* Hudson *et al.* reported that coriandrin exhibits antiviral activity in the presence and absence of UVA against a variety of enveloped viruses. Especially, coriandrin showed activity against HIV-1 *in vitro*.[86] Coriandrin was found to be much more photoreactive than psoralen in some phenomena(*e.g.*, phototoxicity, photo-mutagenesis and clastogenesis). But coriandrin doesn't afford psoralen-DNA adduct and doesn't photosensitize human skin unlike 8-methoxypsoralen. They suggested that viral membrane damages are involved in the antiviral activity. Coriandrin, like α-terthienyl, was phototxic to two membrane-containing animal viruses, murine cytomegalovirus(MCMV) and sindbis virus(SINV). Viruses without membranes were completely resistant to coriandrin. HIV-1, which contains a single-stranded RNA genome plus a membrane, is much less sensitive than SINV to coriandrin+UVA. The approximate order of susceptibility is SINV»MCMV>HIV-1»poliovirus.

The relative photoreactivity of psoralen derivatives against DNA containing virus MCMV is 8-MOP> dictamine>visnagin»angelicin >khellin.

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