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PHOTOINHIBITION OF *Candida albicans* GROWTH BY PSORALEN DERIVATIVES

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Abstract – *Candida albcans* growth inhibition experiments were carried out to determine the phototoxicity of new monofunctional psoralen derivatives, PzPs and HMPzPs, as well as well known 8-MOP and 5-MOP. Although 8-MOP and 5-MOP showed distinct phototoxicity, PzPs and HMPzPs did not inhibit the growth of *Candida albicans* strain, indicating that PzPs derivatives were not good candidate for the treatment of psoriasis. However, vitiligo could be treated by PzPs derivatives without severe phototoxicity because psoralens are known to treat vitiligo by stimulating melanogenesis on skin.

INTRODUCTION

Photosensitizing furocoumarins (psoralens) have been widely used in the photochemotherapy of psoriasis and vitiligo (PUVA; Psoralen + UVA).[1] Recent development of extracorporeal irradiation of 8-methoxypsoralen (8-MOP)[2] containing blood for the treatment of cutaneous T-cell lymphoma and other T-cell mediated diseases such as pemphigus vulgaris, psoriatic arthropathy and chronic lymphocytic leukemia has widened the application of PUVA therapy.[3] 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.[4] For safe blood transfusion, 8-MOP has recently been used to inactivate various types of viruses and bacteria such as animal virus, hapatitis B and C, vesicular stomatis virus in the blood.[5] These photomedicinal and photobiological activities have been associated with the interaction of psoralens with DNA, cross-linking of DNA in particular, on irradiation.[6] However, undesirable side effects such as carcinoma development, erythema formation and hyperpigmentation have also been reported.[7]

Psoralens have two photochemical reaction sites, 3,4-pyrone double bond and 4',5'- furan double bond, both of which are engaged in cross-linking of DNA. The cross-linking of DNA proceeds *via* the following three steps: (i) intercalation complex formation in the dark (ii) photochemical 4',5'- or 3,4- monoadduct formation with the 5,6-double bond of pyrimidine bases of one strand of DNA, followed by (iii) photochemical cross-link formation through the 3,4 -double bond of 4',5'- monoadduct with the other DNA strand.

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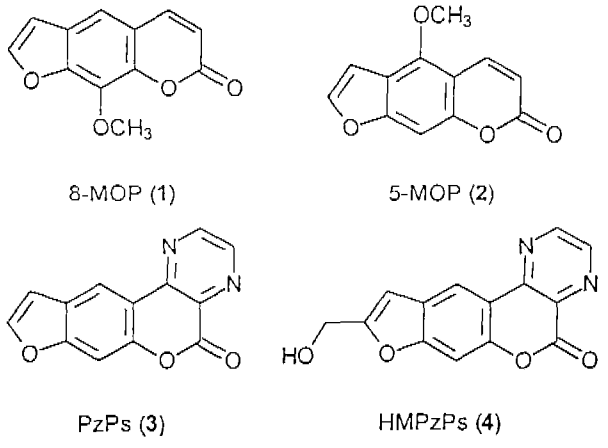
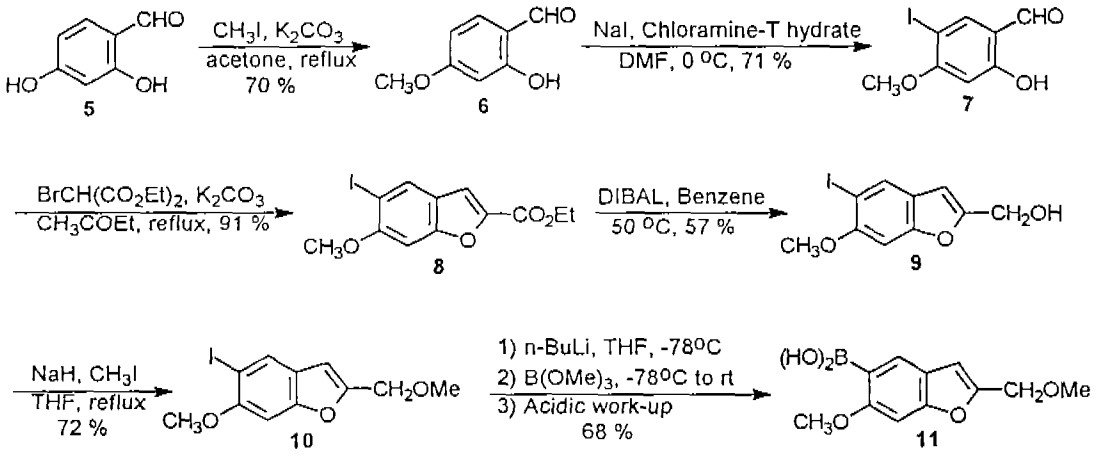


Figure 1. The molecular structure of 8-MOP, 5-MOP, PzPs and HMPzPs.

We have recently reported the synthesis[8], photophysical properties[9], and photoreaction[10] of a new monofunctional psoralen, pyrazinopsoralen (PzPs) (Fig. 1) which has a pyrazine ring fused onto the pyrone double bond of psoralen. PzPs is expected to form [2+2] photocycloaddition products with pyrimidine bases through its 4',5'-furan double bond and this is supported by the photoreaction of PzPs with simple olefins which gave the 4',5'-monoadducts.[10] PzPs is expected to be less phototoxic than other bifunctional psoralen derivatives due to the lack of cross-linking of DNA by blocking one of the reaction sites of psoralens.

Candida albicans growth inhibition method is well-established technique for detecting phototoxicity of drugs *in vivo*.[11] This test is widely used because it is inexpensive, fairly rapid and especially sensitive for the reactions involving DNA damage.[12]



Scheme 1. The synthesis of 5-(6-methoxy-2-methoxymethylbenzofuranyl) boronic acid **11**.

MATERIALS AND METHODS

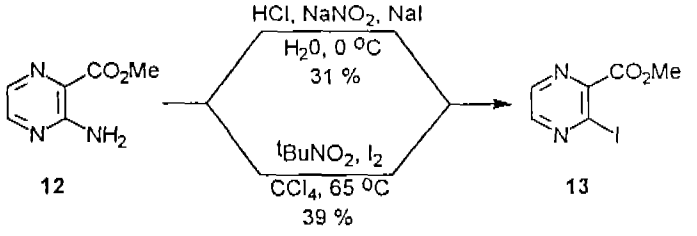
^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AM-300 and AM-200 MHz spectrometers. Proton chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS), and ^{13}C resonances were recorded using the 77.0 ppm $CDCl_3$ resonance of the solvent as an internal reference and reported in ppm downfield from TMS. Infrared (FTIR) spectra were recorded on a Bomem MB-100 Series FTIR spectrophotometer or EQUINOX55 FTIR spectrophotometer (Bruker Co.). Mass spectra were determined with a VG Autospec-Ultma GC/MS spectrometer by the electron impact (EI) method.

Meterials. 8-MOP and 5-methoxypsoralen (5-MOP) were purchased from Aldrich Co. and used without further purification. PzPs and 5'-hydroxymethylpyrazinopsoralen (HMPzPs) were synthesized by the reported method (Scheme 1, 2 and 3).[8] All the reactions were run under dry nitrogen or argon atmosphere in oven-dried glasswares, unless specified otherwise. Reagent grade THF and benzene were distilled from sodium benzophenone ketyl under nitrogen immediately prior to use. Merck precoated silica gel plates (Art. 5554) with fluorescent indicator were used as analytical TLC. Gravity column chromatography and flash chromatography were carried out on silica gel (230-400 mesh from Merck) columns.

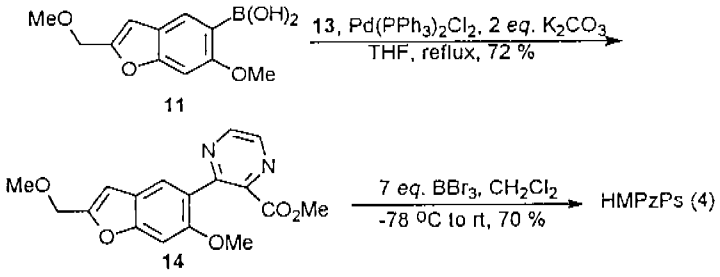
*2-Hydroxymethyl-5-iodo-6-methoxybenzofuran (**9**).* 1.0 *M* DIBAL in toluene (71.7 mL, 71.7 mmol) was added to **8** (Synthesized by the similar method with the literature[13]) (11.29 g, 32.6 mmol) in benzene at room temperature. The reaction mixture was heated to 50°C and kept overnight. To quench the remaining DIBAL, methanol was added at 0°C, the solid formed was filtered off, and the solvent was removed *in vacuo* from filtrate. The purification of filtrate was carried out by column chromatography (Hexane/EtOAc; 1/1) to give yellow liquid **9** (5.65 g, 18. 6 mmol) in 57% yield. ^{1}H-NMR (200 MHz, DMSO-d_6), δ (ppm) 7.94 (s, 1H, aryl-H), 7.14 (s, 1H, aryl-H), 6.60 (s, 1H, furanyl-H), 4.64 (s, 2H, -O<u>*CH*</u>$_2$OH) and 3.89 (s, 3H, methoxy). ^{13}C-NMR (50 MHz, DMSO-d_6), δ (ppm) 159.1, 156.8, 156.2, 131.2, 124.7, 112.2, 103.0, 95.7, 80.2, 57.6 and 57.0. IR (NaCl) cm^{-1} 3363 (OH), 1604 and 1468. EIMS m/e 304 (M^+, 100 %), 287 (M^+-CH_3, 81 %) and 243 (63%).

*5-Iodo-6-methoxy-5-methoxymethylbenzofuran (**10**).* To 670 mg (27.9 mmol) of NaH immersed in mineral oil (55%) in THF was added **9** (5.65 g, 18.6 mmol) in THF at room temperature under Ar. After reflux for 1 h, iodomethane (1.9 mL, 29.8 mmol) was added dropwise and refluxed for 5 h. After cooling, the reaction mixture was extracted with diethyl ether and the extract was dried with anhydrous $MgSO_4$. The column chromatography of residue with hexane-EtOAc (3/1) gave white solid **10** (4.25 g, 13.4 mmol) in 72 % yield. ^{1}H-NMR (200 MHz, CDCl3), δ (ppm) 7.90 (s, 1H, aryl-H), 6.96 (s, 1H, aryl-H), 6.54 (s, 1H, furanyl-H), 4.48 (s, 2H, -O<u>*CH*</u>$_2$OCH$_3$), 3.87 (s, 3H, methoxy) and 3.39 (s, 3H, -OCH$_2$O<u>CH$_3$</u>). ^{13}C-NMR (50 MHz, $CDCl_3$), δ (ppm) 156.3, 155.6, 153.9, 130.7, 123.4, 104.7, 94.8, 80.1, 66.7, 58.2 and 56.6. IR (NaCl) cm^{-1} 2926, 1613 and 1468. EIMS m/e 318 (M^+, 60%), 287 (M^+-OCH_3, 100%), 272 (M^+-OCH_3-CH_3, 63%) and 243 (46%).

*5-(6-Methoxy-2-methoxymethylbenzofuranyl)boronic acid (**11**).* 11 was synthesized by the same method used in PzPs synthesis[8] in 68% yield as white solid. ^{1}H-NMR (200 MHz, $CDCl_3$+CD_3OD),



Scheme 2. The synthesis of methyl 3-iodopyrazine 2-carboxylate **13**.



Scheme 3. The synthesis of HMPzPs.

δ (ppm) 7.75 (s, 1H, aryl-H), 6.80 (s, 1H, aryl-H), 6.44 (s, 1H, furanyl-H), 4.31 (s, 2H, -O<u>*CH*</u>$_2$OCH$_3$), 3.96(br, 2H, B(OH)$_2$), 3.70 (s, 3H, methoxy) and 3.21 (s, 3H, -OCH$_2$O<u>*CH*</u>$_3$). ^{13}C-NMR (50 MHz, CDCl$_3$+CD$_3$OD), δ (ppm) 162.4, 157.7, 152.9, 129.0, 128.6, 121.2, 105.7, 93.3, 66.3, 57.4 and 55.3.

Methyl 3-iodopyrazine 2-carboxylate (13). *Tert*-butyl nitrite (7.76 mL, 65.3 mmol) was slowly added to CCl_4 solution of amide **12** (5.00 g, 32.7 mmol) and iodine (4.97 g, 19.6 mmol) at 65°C. After stirring at that temperature for 3 h, the reaction mixture was cooled and extracted with methylene chloride. The extract was washed with 10% aqueous sodium thiosulfate solution and water. Dried extract was purified by column chromatography using hexane-EtOAc (2/1) as an eluent, giving yellow solid **13** (3.34 g, 12.7 mmol) in 39% yield. The spectral data were same as those reported previously.[8] This reaction was more convenient and gave the same iodide **13** in better yield than the classical Sandmeyer reaction used previously[8] (Scheme 2). The use of organic solvent and non-acidic condition may prevent the hydrolysis of methyl ester, which would give the iodinated products in better yield.

Methyl 3-(6-methoxy-2-methoxymethylbenzofuran-5-yl)pyrazine 2-carboxylate (14). Boronic acid **11** (987 mg, 4.18 mmol), iodide **13** (1.33 g, 5.01 mmol) and Pd(PPh$_3$)$_2$Cl$_2$ in THF was heated to 70°C for 30 min and deoxygenated 2 *M* aqueous K_2CO_3 solution (4.18 mL, 8. 36 mmol) was added slowly. After stirring at 80°C for 5 h, the reaction mixture was extracted with EtOAc and dried with anhydrous $MgSO_4$. The purification by column chromatography (Hexane-EtOAc, 1/1) gave the coupling product **14** (974 mg, 3.01 mmol) in 72% yield. ^{1}H-NMR (200 MHz, CDCl$_3$), δ (ppm) 8.66 (d, 2.39 Hz, 1H, pyrazinyl-H), 8.46 (d, 2.39 Hz, 1H, pyrazinyl-H), 7.73 (s, 1H, aryl-H), 6.95 (s, 1H, aryl-H), 6.61 (s, 1H, furanyl-H), 4.44 (s, 2H, -O<u>*CH*</u>$_2$OCH$_3$), 3.74 (s, 3H, methoxy), 3.67 (s, 3H, methoxy) and 3.33 (s, 3H,-OCH$_2$O<u>*CH*</u>$_3$). ^{13}C-NMR (50 MHz, CDCl$_3$), δ (ppm) 165.9, 156.8, 154.5, 153.6, 151.4, 145.4, 145.3, 140.9, 132.3, 122.8, 121.4, 105.8, 93.8, 66.5, 57.8, 55.1 and 52.2. EIMS m/e 328 (M$^+$, 89 %), 297 (M$^+$-OCH$_3$, 100 %), 269 (62 %) and 223 (74 %).

5'(-Hydroxymethylpyrazinopsoralen, (HMPzPs) (4). **4** was synthesized from **14** by the same method used in PzPs synthesis[8] in 70% yield as yellow solid. ^{1}H-NMR (300 MHz, DMSO-d$_6$+CDCl$_3$), δ (ppm) 8.62 (d, 2.03 Hz, 1H, pyrazinyl-H), 8.48 (d, 2.03 Hz, 1H, pyrazinyl-H), 8.31 (s, 1H, aryl-H), 7.10 (s, 1H, aryl-H), 6.56 (s, 1H, furanyl-H), 4.97 (s, 1H, -OCH$_2$O<u>*H*</u>) and 4.27 (s, 2H, -O<u>*CH*</u>$_2$OH). ^{13}C-NMR (75 MHz, DMSO-d$_6$+CDCl$_3$), δ (ppm) 157.9, 156.2, 154.4, 149.5, 149.1, 147.5, 144.8, 131.9, 125.2, 116.7, 113.8, 105.3 and 99.1. EIMS m/e 268 (M$^+$, 42%), 266 (M$^+$-2H, 44%), 251 (M$^+$-OH, 100%) and 223 (M$^+$-H-CO$_2$, 49%).

Method. 8-MOP, 5-MOP, PzPs and HMPzPs were dissolved in dimethyl sulfoxide (DMSO). The concentrations of solutions were 0.1, 0.3 and 1.0% (g/v). Four filter paper discs, each set containing prepared solutions at the same concentration, were placed in a petri dish in which fresh culture from a *Candida albicans* stock strain was suspended on a Sabouraud glucose agar. The same amount of control DMSO was also placed in the center of petri dishes. Each petri dish was irradiated with UVA (20, 40 and 80 J/cm^2). One series was cultured without irradiation for controls. After 48 h, the *Candida*-free zone around the disc was checked to determine the phototoxicity of drugs.

RESULTS AND DISCUSSION

Limited solubility of PzPs toward water as well as organic solvents is expected to be a major problem in its application to clinics. To afford desirable solubility, hydroxymethyl group was introduced at 5'-position of PzPs, and the water solubility of resulting 5'-hydroxymethylpyrazinopsoralen (HMPzPs) was measured in comparison with that of PzPs. The solubility increased from 65μM for PzPs to 90μM for HMPzPs.

There are many methods to determine the phototoxicity of a drug. Care must be taken to determine method(s) because each method has peculiar characteristics and different drugs have different phototoxicity mechanisms. *In vitro* methods include *Candida albicans* growth inhibition, photohemolysis test, *Salmonella typhimurium* mutant strain method[14], and so on. *In vivo* methods include test of erythema in mouse or guinea pig skin, and mouse tail technique[15]. *Candida albicans* growth inhibition method is well-established and widely used technique because it is inexpensive, fairly rapid and especially sensitive for the reactions involving DNA damage, which makes this test suitable to get insight into the phototoxicity of psoralens. It is generally agreed that mechanism of phototoxicity is not singular, but depends on the interactions of at least three different damages occurring in the skin: nuclear damage, cytoplasm and/or cell membrane damage and injury to cell constituents. *Candida albicans* growth inhibition technique may not reflect all of these three domages.

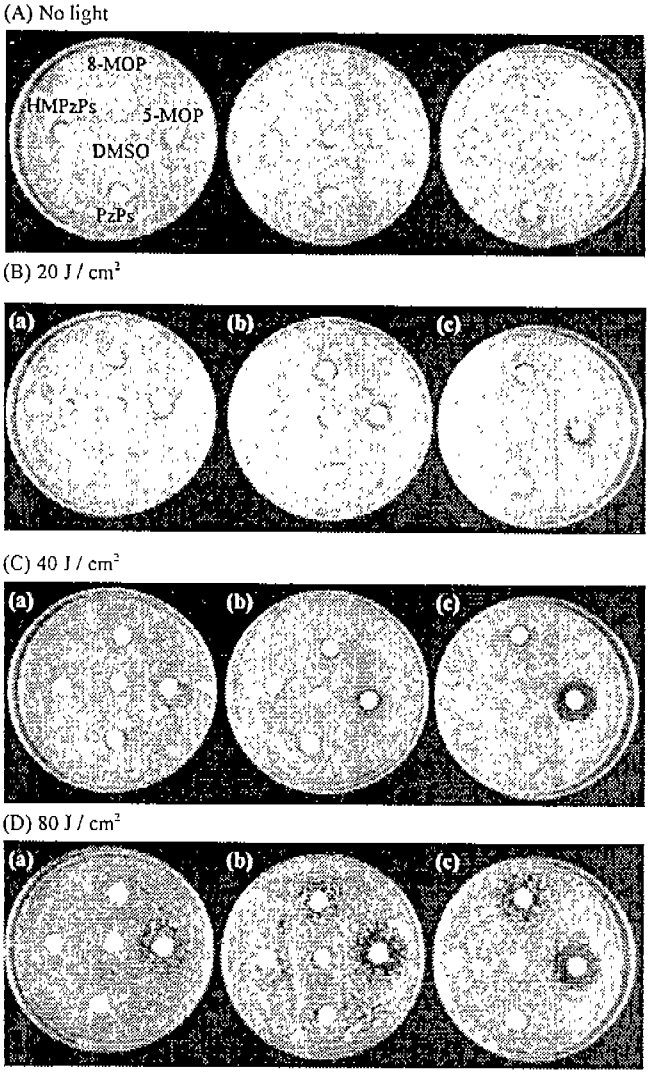


Figure 2. The phototoxicity of psoralen derivatives. Each petri dish contains five filter papers that have aliquot of 8-MOP, 5-MOP, PzPs and HMPzPs solutions. The UVA intensities were 0 (A), 20 (B), 40 (C) and 80 J/cm^2 (D). The concentrations of each drug were 0.1 (a), 0.3 (b) and 1.0 % (g/v).

Phototoxicity of well-known psoralens, 8-MOP and 5-MOP, and newly synthesized PzPs and HMPzPs was determined using *Candida albicans* strain as shown in Fig. 2. It was first reported by Daniels that psoralens are phototoxic to *Candida albicans*.[11] Horikawa *et al.* also reported the phototoxicity of 8-MOP in comparison with other drugs.[12b] The DMSO only used as a control did not show the *Candida*-free zone in either irradiated or non-irradiated plate indicating that DMSO with or without light did not inhibit the growth of *Candida albicans*. The treatment of drugs in the dark did not show the growth inhibition [Fig. 2(A)]. 5-MOP and 8-MOP showed evident *Candida*-free zone, the area of which is increased by increasing the drug concentration and light intensity. However, PzPs and HMPzPs even with the highest concentration and the most intensive light intensity (1.0% and 80 J/cm^2) used.

The absence of phototoxicity implies the inefficient photoreaction of PzPs or HMPzPs with DNA. It seems that PzPs and HMPzPs are not good candidates for the treatment of psoriasis that is characterized by excessive proliferation of DNA in skin. Psoralen has been known to inhibit the proliferation of DNA of psoriatic cells, resulting in the treatment of psoriasis. However, other skin disease, vitiligo, may be cured by PzPs or HMPzPs without severe phototoxicity since the mechanism of the treatment of vitiligo does not involve DNA damage.

Vitiligo is characterized by the inefficient formation of pigments on the skin. Treatment of vitiligo by psoralens is known to be due to the stimulation of melanocytes and keratinocytes that involve in the formation of pigments in the skin. Although psoralens inhibit the proliferation of melanocytes by forming DNA adducts in the initial stage of cell cycle, psoralens can induce pigmentation by activating tyrosinase, the enzyme catalyzing the formation of pigment in G2 phase of the cell cycle. Several other mechanisms have also been suggested to be involved in the activation of melanocyte. First, UVA light, with or without psoralens, directly stimulates the proliferation of melanocyte.[16] Secondly, PUVA may act on epidermal keratinocyte or dermal components to stimulate them to release certain melanocyte growth stimulation factors.[17] Thirdly, PUVA immunologically leads to the impairment of epidermal Lagerhans cell function and alteration of circulating T and B cell functions, which results in the suppression of the stimulus for melanocyte destruction.[18] For more concerns, the report by Pathak *et al.* is preferable.[19] Although molecular basis on the treatment of vitiligo by psoralens remains to be elucidated, the pathogenesis of vitiligo is related with autoimmune system.[20] Therefore, treatment of vitiligo by psoralens is expected to be associated with immunology unlike psoriasis. Although vitiligo can be treated only by PUVA, psoriasis can be treated by UVB as well as PUVA, indicating that inhibition of DNA synthesis of melanocyte is major treatment mechanism of psoriasis by T-T dimer (in UVB) and T-psoralen-T crosslink (in PUVA). The studies related with the pigmentation of skin and other kinds of *in vitro* and *in vivo* test using PzPs and HMPzPs are in progress.

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2. Abbreviation Used: 8-MOP, 8-methoxypsoralen; 5-MOP, 5-methoxypsoralen; PzPs, pyrazinopsoralen, HMPzPs, 5'-hydroxymethylpyrazinopsoralen; EIMS, electron impact mass spectra; br, broad band.
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