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NOTES

Preparation and Skin-Photosensitizing Activity of Substituted Psoralens

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Abstract □ 8-Monosubstituted and 5,8-disubstituted psoralen derivatives were prepared, and their skin-photosensitizing activity was evaluated. The results were correlated in terms of molecular configuration, and 8-allyloxypsoralen can be considered as a new agent of potent photodynamic activity.

Keyphrases □ Psoralens, substituted—synthesized, evaluated for skin-photosensitizing activity □ Photosensitizing activity, skin—various substituted psoralens evaluated □ Structure–activity relationships—various substituted psoralens evaluated for skin-photosensitizing activity

In a continuation of previous studies (1–5), this report describes the preparation and skin-photosensitizing activity of substituted psoralens. The synthesis and photosensitizing activity of many psoralen (I) derivatives have been investigated extensively (6, 7), but little has been published concerning the 5,8-disubstituted psoralens. For example, while 5-methylpsoralen and 8-methylpsoralen are potent skin photosensitizers (8), nothing has been reported about 5,8-dimethylpsoralen. Furthermore, it was observed (9) that allyloxycoumarins have weak, but definite, photodynamic activity. Nevertheless, the effect on the photodynamic activity of introducing allyl, allyloxy, or dimethylallyl substituents in the psoralen molecule has not been studied.

On the other hand, the structure–activity relationships in the furocoumarin series demonstrated clearly the following. 2-Thiopsoralen derivatives and oxazolocoumarins do not exhibit any photodynamic activity (6). There is great variation in the potency of the skin-photosensitizing activity of the derivatives, determined by the number and the position of methyl groups (8). Methyl substitution at the 5′-carbon of psoralen does not alter the activity, but methyl substitution at the 4′-position results in a considerable loss of activity (9). Substitution with a methoxy group at the 5- or 8-carbon results in decreased activity, while nitro or amino group substitution renders the compound completely inactive (9). The fact that 2,6,8-trimethylbenzodifuran (10) and some other coumarin derivatives (9) do elicit photosensitized responses suggests that the furocoumarin structure is not essential for photosensitizing activity.

Further investigation is needed to reveal definite points in the structure–activity relationship in the furocoumarin series.

DISCUSSION

Previously, the isolation of xanthotoxin (II) and imperatorin (III) from the fruits of the umbellifer *Ammi majus* (L.)[1] was reported (1). These compounds were readily transformed into xanthotoxol (IV) according to a reported (4) procedure. The identity of xanthotoxin, imperatorin, and xanthotoxol was established through mixed melting-point, UV, IR, and NMR spectral comparisons with authentic samples[1]. For the preparation of derivatives V–VII, the procedure followed was essentially the same as that described previously (3).

It was reported (11) that imperatorin underwent Claisen rearrangement to afford alloimperatorin (IX) by heating in a vacuum. In the present work, to induce the allyl ethers to undergo Claisen rearrangement, the ethers were heated under reflux for 6 hr in ethylene glycol. In this way, 5-allyl-8-hydroxypsoralen (VIII) and IX were obtained in low yields (65%). Better results or even quantitative yields were obtained by refluxing the allyl ethers in *N*,*N*-diethylaniline for 1 hr. In the latter method, Claisen rearrangement products were of higher purity; 1 hr of refluxing was sufficient.

Methylation, acetylation, and allylation reactions were carried out according to the general method of etherification and esterification (3). However, allylation of VIII ended in failure. The identity of the new psoralen derivatives was ascertained by spectral (IR and NMR) analysis.

Compounds V–XIV (Table I) were prepared and screened as dermal photosensitizing agents. Two tests were performed with albino guinea pig skin. The preliminary test, employing very strong conditions (high amount of substance and high amount of radiation), was used for selecting the active compounds, even if weakly active, from the inactive ones. In a second test, only the active compounds were tested in very mild conditions and their photosensitizing activities were compared with that of psoralen.

The relative activity was evaluated by comparing the erythema re-

[1] Provided by Memphis Chemical Co., Cairo, Egypt.

Table I—Data for 8-Monosubstituted and 5,8-Disubstituted Psoralens[a]

Compound	Melting Point	Yield, %	Empirical Formula	Analysis, % Calc.	Found
V	68–70°	95	$C_{14}H_{10}O_4$	C 69.42	69.39
				H 4.13	4.24
VI	175–177°[b]	95	$C_{13}H_8O_5$	C 63.93	63.91
				H 3.28	3.40
VII	194–196°	90	$C_{18}H_{10}O_5$	C 70.59	70.77
				H 3.27	3.44
VIII	203–205°	65[c], 98[d]	$C_{14}H_{10}O_4$	C 69.4	69.24
				H 4.13	4.10
IX	228–230°[e]	63[c], 97[d]	$C_{16}H_{14}O_4$	C 71.11	70.79
				H 5.11	5.08
X	98–100°	90	$C_{15}H_{12}O_4$	C 70.31	69.96
				H 4.70	4.61
XI	158–160°	80	$C_{16}H_{12}O_5$	C 67.61	67.42
				H 4.23	4.32
XII	109–111°[f]	90	$C_{17}H_{16}O_4$	C 71.83	71.88
				H 5.63	5.67
XIII	106–108°	87	$C_{18}H_{16}O_5$	C 69.23	68.98
				H 5.13	5.17
XIV	71–73°	85	$C_{19}H_{18}O_4$	C 73.55	73.88
				H 5.81	5.87

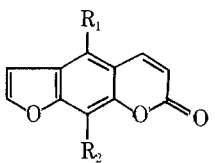
[a] The crystallization solvent was ethanol except for VII for which benzene was used. [b] Lit. (7) mp 178°. [c] Method I. [d] Method II. [e] Lit. (11) mp 233°. [f] Lit. (7) mp 113°.

sponse as observed by visible intensity grading. Introduction of hydroxy (VIII and IX), acetoxy (VI, XI, and XIII), or benzoyloxy (VII) substituents at the 8-position in the psoralen molecule resulted in a marked loss of the photodynamic effect. However, 5,8-disubstituted psoralens (X, XII, and XIV) did not show significant activity. Experimental evidence has now been obtained to confirm that the activity of psoralens is markedly reduced or eliminated upon blocking the 5- and 8-positions, which are the active centers in the psoralen molecule. Moreover, methylation of the hydroxyl group (X and XII) partially restores the photosensitizing activity of psoralen derivatives. Such results are analogous to previous findings (9).

Surprisingly, 8-allyloxypsoralen (V) proved very active; it was only slightly less active than psoralen itself. This result appears to be in contradiction with other reported (6, 9) studies. Accordingly, these data, coupled with the previously mentioned observations, served to confirm that it is desirable to explore the precise structure–activity relationship fully.

EXPERIMENTAL[2]

Claisen Rearrangement—*Method I*—A solution of 5 g of the allyloxypsoralen derivative in 25 ml of ethylene glycol was refluxed for 6 hr. The solution was cooled, and the solid product was filtered and recrystallized from a suitable solvent.



I: $R_1 = R_2 = H$
II: $R_1 = H$, $R_2 = OCH_3$
III: $R_1 = H$, $R_2 = OCH_2CH{=}C(CH_3)_2$
IV: $R_1 = H$, $R_2 = OH$
V: $R_1 = H$, $R_2 = OCH_2CH{=}CH_2$
VI: $R_1 = H$, $R_2 = OCOCH_3$
VII: $R_1 = H$, $R_2 = OCOC_6H_5$
VIII: $R_1 = CH_2CH{=}CH_2$, $R_2 = OH$
IX: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = OH$
X: $R_1 = CH_2CH{=}CH_2$, $R_2 = OCH_3$
XI: $R_1 = CH_2CH{=}CH_2$, $R_2 = OCOCH_3$
XII: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = OCH_3$
XIII: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = OCOCH_3$
XIV: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = OCH_2CH{=}CH_2$

Method II—A solution of the allyl ether in about 25 ml of *N,N*-diethylaniline was refluxed for 1 hr. The precipitated product was collected and purified.

Methylation—A mixture of 2 g of the corresponding crude Claisen rearranged product (VIII or IX), 20 g of anhydrous potassium carbonate, 20 ml of methyl iodide, and 250 ml of dry acetone was refluxed and stirred for 24 hr. The acetone filtrate and washings were evaporated to dryness; the residue, after washing with water, was recrystallized from alcohol.

Acetylation—A mixture of 2 g of VIII or IX and a few crystals of fused sodium acetate in 20 ml of acetic anhydride was heated under reflux for 3 hr and then stirred with water until excess acetic anhydride had decomposed. The insoluble product was filtered and recrystallized from alcohol.

Allylation—A mixture of 2 g of the 8-hydroxypsoralen derivative (VIII or IX), 10 g of anhydrous potassium carbonate, 10 ml of allyl bromide, and 250 ml of dry acetone was stirred and refluxed for 20 hr. Evaporation of the acetone filtrate under reduced pressure left a residue, which was washed thoroughly with water, dried, and washed once with petroleum ether (bp 30–60°) to remove excess allyl bromide. The residue was then dried and recrystallized from the appropriate solvent.

Table I lists the psoralens (V–XIV) with their physical constants. Their identity was established by UV, IR, and NMR spectral properties.

Skin-Photosensitizing Activity—*Animals*—Common guinea pigs were used; the hair was removed from the abdomen with scissors. Exact amounts of an acetone solution of the compound to be tested were then placed on 4 cm^2 (2 × 2 cm) of nonpigmented skin, and rapid evaporation of the acetone was accomplished.

Irradiation—A 500-w solar lamp[3] was used; it emitted strongly at 365 nm and in the visible region.

Preliminary Pharmacological Test—A concentration of 50 $\mu g/cm^2$ of a compound was tested with the lamp at a distance of 25 cm, an irradiation time of 60 min, and observation after 24 hr. Compounds VI–IX, XI, and XIII were inactive. Compounds X, XII, and XIV were slightly active. In contrast, V was very active.

Comparative Screening with Psoralen—Only V, X, XII, and XIV were tested at 2.5 $\mu g/cm^2$ with the lamp at 45-cm distance, irradiation for 60 min, and observation of erythema after 24 hr. In these pharmacological tests, the compounds were placed on square areas of the abdomen of the same animal. The tests were repeated on five different animals with the same results. Compared to I, which was very active (3+ erythema), V was also very active (2+ ± erythema), X and XII were weakly active (1+ erythema), and XIV was very weak (± erythema).

REFERENCES

(1) M. A. Loutfy, M. A. Hassan, and H. A. Abu-Shady, *Pharmazie*, **30**, 748 (1975).

[2] Melting points were determined in capillary tubes heated in a Mel-Temp apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer model 621 spectrophotometer from samples in potassium bromide disks. UV absorption spectra were measured with a Perkin-Elmer 124 double-beam spectrophotometer. NMR spectra were determined with a Varian model A-60A spectrometer. All IR and NMR spectra of the new compounds were in agreement with assigned structures. Elemental analyses were performed by Dr. F. B. Strauss, Oxford, England.

[3] Osram HWA.

(2) M. A. Loutfy and H. A. Abu-Shady, *ibid.*, **31**, 573 (1976).
(3) M. A. Loutfy, M. A. Hassan, A. I. Jado, and H. A. Abu-Shady, *ibid.*, **31**, 819 (1976).
(4) M. A. Loutfy, Ph.D. thesis, University of Riyadh, Riyadh, Saudi Arabia, 1976.
(5) M. A. Loutfy and H. A. Abu-Shady, *Pharmazie*, **32**, 240 (1977).
(6) L. Musajo and G. Rodighiero, "Photophysiology," vol. VII, A. C. Giese, Ed., Academic, New York, N.Y., 1972, p. 115.
(7) A. Mustafa, "Furopyrans and Furopyrones," Interscience, London, England, 1967, p. 14.
(8) F. Dall'Acqua, S. Marciani, D. Vedaldi, and G. Rodighiero, *Biochim. Biophys. Acta*, **353**, 267 (1974).
(9) M. A. Pathak, J. H. Fellman, and K. D. Kaufman, *J. Invest. Dermatol.*, **35**, 165 (1960).
(10) L. R. Worden, A. W. Bergstahler, K. D. Kaufman, J. A. Weis, and T. K. Schaaf, *J. Heterocycl. Chem.*, **6**, 191 (1969).
(11) A. Schonberg and A. Sina, *J. Am. Chem. Soc.*, **72**, 4827 (1950).

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High-Speed Liquid Chromatographic Determination of Pilocarpine in Pharmaceutical Dosage Forms

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Abstract □ A specific method for the direct determination of pilocarpine in aqueous pharmaceuticals in the presence of decomposition products, methylcellulose, and other ingredients usually present in pharmaceuticals is described. The method involves separation by high-speed liquid chromatography using, in series, octadecylsilane bonded to silica and cyanopropylsilane bonded to silica columns and a tetrahydrofuran–pH 9.2 borate buffer (3:7) eluant. Quantitation is achieved by monitoring the absorbance of the effluent at 254 nm and using a pyridine internal standard and a calibration curve prepared from known concentrations of pilocarpine nitrate. The reproducibility of the retention time and peak area was better than 2.0%.

Keyphrases □ Pilocarpine—high-speed liquid chromatographic analysis, pharmaceutical preparations □ High-speed liquid chromatography—analysis, pilocarpine in pharmaceutical preparations □ Ophthalmic cholinergics—pilocarpine, high-speed liquid chromatographic analysis, pharmaceutical preparations

Many methods for estimating pilocarpine and its salts are available. Several are based on the titration of the extracted base with standard acid, with bromphenol blue (1–3), methyl red (4, 5), methyl orange (6), or dimethyl yellow (7) as the indicator. Several gravimetric (8–10), colorimetric (11), and iodometric (12) methods were developed. Recently, a GLC method using electron-capture detection was employed (13).

The USP XIX monographs for pilocarpine hydrochloride (14) and pilocarpine nitrate (15) incorporate a nonaqueous titration. However, the monographs on pilocarpine hydrochloride ophthalmic solution (15) and pilocarpine nitrate ophthalmic solution (16) require an extraction procedure, followed by colorimetric determination. Most of these methods lack the specificity and simplicity desired in routine analysis, especially in the presence of degradation products and/or other ingredients. In addition, the extraction of pilocarpine base from pharmaceuticals followed by colorimetric determination leads to erroneous results[1].

Recently, high-speed liquid chromatography (HSLC) was used to analyze alkaloids (17, 18). This paper describes an HSLC method that permits the quantitative determination of pilocarpine in the presence of its degradation products and other chemicals commonly included with pilocarpine salts in pharmaceutical products.

Table I—Calibration Data[a] for HSLC Pilocarpine Analysis

Concentration Added, mg/ml	Peak Area of Pilocarpine / Peak Area of Standard
5	0.141 ± 0.0065[b]
10	0.286 ± 0.0104
20	0.562 ± 0.0084
30	0.830 ± 0.0080

[a] Average of 10 replicate injections. [b] Standard deviation.

EXPERIMENTAL

Apparatus—A high-speed liquid chromatograph[2] equipped with a pump[3], a single-wavelength UV monitor (254 nm), and a liquid chromatograph injector[4] was used.

Columns—An octadecylsilane bonded to silica[5] column and a cyanopropylsilane[6] column were used in series. Both columns were 300-mm long × 4-mm i.d. stainless steel tubes.

Reagents—Sodium borate, tetrahydrofuran, and pyridine were reagent grades. Pilocarpine nitrate USP was used without further purification.

[1] U.S. Pharmacopeia, Drug Standards Division, personal communication.
[2] Model 202, Waters Associates.
[3] M-6000.
[4] U6K Universal.
[5] μBondapak C_{18}, Waters Associates.
[6] μBondapak CN, Waters Associates.