PHOTOREACTION OF N-BUTYL 3,4-DIMETHOXY-6-NITROBENZAMIDE WITH BUTYLAMINE. A MODEL STUDY FOR LYSINE-DIRECTED PHOTOAFFINITY LABELLING

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Ultraviolet irradiation of the title compound *I* in the presence of butylamine gave predominantly products of nucleophilic photosubstitution by the amine, *i.e.*, nitroanilines *IIa* and *IIb*. Besides, small amounts of products of hydrolysis (phenol *III*) and reductive coupling (azoxybenzene *IV*) were also formed. Comparison of the overall photolysis rate of *I* with that of 3,4-dimethoxy-1-nitrobenzene (*V*) indicates a minor loss of reactivity, most probably due to some deviation from coplanarity of the activating nitro group and the aromatic ring.

In the course of our studies towards the design of an effective lysine-directed photoaffinity probe we have recently found that 3,4-dimethoxy-1-nitrobenzene (V), one of the promising photolabels[1,2], exhibits a pronounced pH dependence of regioselectivity in the photoreaction with butylamine[3]. Moreover, the selectivity is rather low[3,4], especially in the pH region typical for biological systems. Therefore, the previously devised modes of attachment of the 3,4-dialkoxy-1-nitrobenzene moiety to a biomolecule, namely through the ether link[1,2], can be *a priori* classified as not very advantageous. An attempted photoaffinity labelling of a lysine-containing biomolecule, using a photoreactive ligand of the type $R—O—C_6H_3(OCH_3)NO_2$, would inevitably lead to simultaneous label transfer and crosslinking.

This difficulty could be overcome by exploiting a derivative of the type $R—O—C_6H_4NO_2$, since both 3- and 4-methoxy-1-nitrobenzenes were reported to react with butylamine upon UV irradiation[4]. Obviously, such systems would undergo exclusive label transfer. We have tentatively explored this possibility, but the preliminary experiments had shown a pronounced decrease of the overall reactivity in comparison with V, as well as formation of large amounts of side-products.

Another way to circumvent the problem of low selectivity in the case of 3,4-dialkoxy-1-nitrobenzenes is based on the approach presented in this paper; here we propose to attach the photoreactive moiety to its carrier molecule through N-substituted carbamoyl link. The introduction of such a handle into the molecule of V is easy to achieve, starting from the readily available 3,4-dimethoxy-6-nitrobenzoic acid. It was the aim of the present study to establish what influence will have the

introduction of the RNHCO group upon the overall reactivity, with respect to possible sterically induced deviations from coplanarity of the aromatic ring and the essential nitro substituent.

UV irradiation of either *I* or *V* with excess butylamine in aqueous tert-butanol at pH 11·0 is accompanied by spectral changes depicted in Fig. 1. All isosbestic points are preserved up to the conversion of approximately 90%. This clearly indicates that the photoreactions of both compounds are well-defined processes, occuring either as simple A → B type transformations or, more probably, as sets of parallel reactions with constant branching ratios. A comparison of the reaction rates under identical irradiation conditions, as measured by the appearance of the absorption bands near 270 nm, is illustrated on Fig. 2. It follows that there is only a minor difference in reactivity between *I* and the reference substrate *V*. The small decrease of the overall reaction rate can be most probably ascribed to the steric interactions mentioned above.
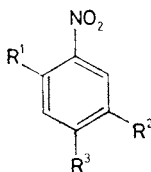
I, $R^1 = C_4H_9NHCO$; $R^2 = OCH_3$; $R^3 = OCH_3$
IIa, $R^1 = C_4H_9NHCO$; $R^2 = NHC_4H_9$; $R^3 = OCH_3$
IIb, $R^1 = C_4H_9NHCO$; $R^2 = OCH_3$; $R^3 = NHC_4H_9$
III, $R^1 = C_4H_9NHCO$; $R^2 = OH$; $R^3 = OCH_3$
V, $R^1 = H$; $R^2 = OCH_3$; $R^3 = OCH_3$
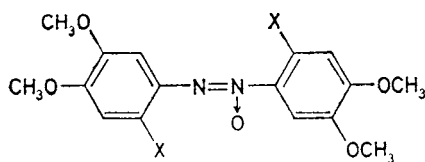
IV, $X = C_4H_9NHCO$

Preparative photolysis of *I* in the presence of butylamine yielded predominantly nitroanilines *IIa* and *IIb*. Besides, phenol *III* and azoxybenzene *IV* were also formed in moderate yields. Regardless of the relatively high conversion of the starting compound *I* (86%), the amount of unidentified by-products was rather low (*IIa* 32%, *IIb* 18%, *III* 21%, and *IV* 5%). This indicates that, despite of the presence of the *ortho*-nitro substituent, the undesired intramolecular photoreactions are ineffective.

The formation of the azoxybenzene *IV* as a side-product does not lower the usefulness of *I* as a potential photoaffinity label, since any interaction of two or more photo-probe molecules, attached to a biopolymer, is rather improbable.

We can conclude that the introduction of the N-alkylcarbamoyl side-chain into the 3,4-dimethoxy-1-nitrobenzene molecule leads to an unimportant loss of the overall reactivity. The slightly enhanced yield of the phenol *III* (21%), in comparison with that of 2-methoxy-5-nitrophenol formed in the photolysis of *V* under identical
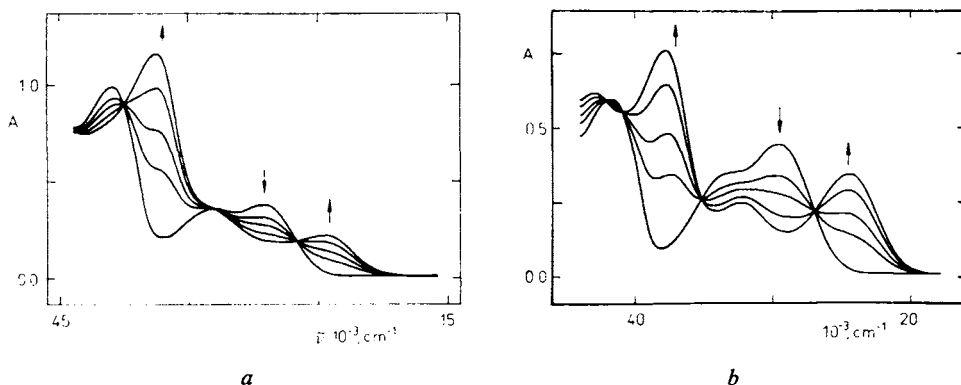

a	*b*

FIG. 1

Spectral changes caused by the irradiation of a : I (6·8 . 10^{-5} mol l^{-1}), b: V (6·1 . 10^{-5} mol l^{-1}), in 50% aqueous tert-butanol, borate buffer (0·025 mol l^{-1}; pH 11·0), light source — HBO-200 mercury lamp with a Pyrex filter. Spectra were taken at a: 0·0; 1·5; 3·0; 6·0; 12·0 min, b: 0·0; 1·0; 2·0; 5·0; 10·0 min
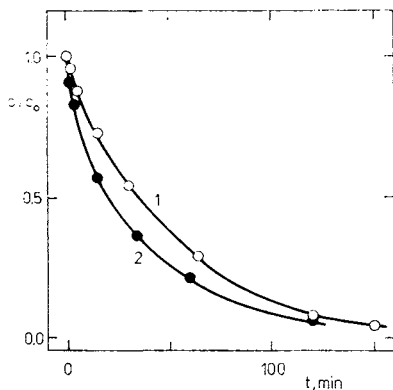

FIG. 2

Time dependence of the actual-to-initial concentration ratio c/c_0 for the compounds *V* (curve 1) and *I* (curve 2) during the photolysis. For the conditions see Experimental

conditions[3] (11%), indicates that *I* is somewhat more prone to the undesired competing photohydrolysis. However, this is outweighed by the principal advantage of *I*, *i.e.*, its exclusive photocrosslinking ability.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. UV spectra were recorded with a Varian Cary 219 spectrophotometer in methanol. ^{1}H NMR spectra were measured with a Varian XL-200 instrument in deuteriochloroform using tetramethylsilane as an internal standard; chemical shifts are in δ units and the interaction constants in Hz. High-resolution mass spectra were measured with an AEI MS-902 spectrometer, electron energy 70 eV, source temperature 150—250°C. IR spectra were taken on a UR-20 (Zeiss) instrument in chloroform; positions of the absorption maxima are given in cm^{-1}.

Analytical high-performance liquid chromatography was performed on the modified silica gel Separon SI C-18 (Laboratorní přístroje, Praha), particle size 5 μm, column 25 × 0·4 cm, elution with methanol–water 7 : 3, flow rate 0·5 ml min^{-1}, UV detection at 365 nm. Preparative HPLC was run under the same conditions except for 20 μm sorbent particle size and 4·0 ml min^{-1} flow rate. Thin-layer chromatography was performed on the Merck type 60 silica gel GF-254.

N-Butyl-3,4-dimethoxy-6-nitrobenzamide (*I*)

3,4-Dimethoxy-6-nitrobenzoyl chloride[5] (1·46 g; 5 mmol) and butylamine (3·65 g; 50 mmol) were refluxed in benzene solution (20 ml) for 60 min. After cooling, the reaction mixture was extracted with 0·5M-HCl (2 × 5 ml) and water (2 × 5 ml), dried with magnesium sulphate, evaporated *in vacuo* and the product was recrystallized from diethyl ether; m.p. 134—135°C, yield 71%. For $C_{13}H_{18}N_2O_5$ (282·2) calculated: 55·30% C, 6·44% H, 9·92% N; found: 55·18% C, 6·48% H, 9·78% N. High-resolution mass spectrum: calculated 282·1216; found 282·1221. IR spectrum: 1 670, 3 440 (CONH), 1 522, 1 337 (NO_2), 1 285 (C_{Ar}—O). ^{1}H NMR spectrum: 7·63 (bs, H-2), 6·90 (bs, H-5), 3·97 (s, OCH_3), 3·98 (s, OCH_3), 5·64 (bt, NH, $J = 5·6, 5·6$), 3·47 (dt, CH_2N, $J = 5·6, 7·2, 7·2$), 1·44 (m, CH_2), 1·64 (m, CH_2), 0·97 (t, CH_3, $J = 7·2, 7·2$). UV spectrum λ_{max}, nm (ε, m^2 mol^{-1}): 340 (562), 299 (532), 243 (1 484).

Preparative Photolysis of *I*

Amide *I* (141 mg; 0·50 mmol) in tert-butanol (200 ml) was mixed with borate buffer (0·1 mol l^{-1}, pH 11·0, 200 ml). Butylamine (1·55 ml; 15·5 mmol) was added, the mixture was deoxygenated with a stream of argon (40 min) and irradiated in a preparative immersion-well photoreactor (Fritz GmbH) with the Pyrex-filtered light of a medium pressure mercury lamp TQ-150 (Hanovia) for 2 h at 20 ± 2°C. The reaction mixture was saturated with sodium chloride, the aqueous layer was extracted with tert-butanol (50 ml) and acetonitrile (50 ml) and the combined extracts were evaporated *in vacuo*. The red-brown residue was dissolved in chloroform (50 ml), the solution was extracted with 0·10M-HCl (2 × 10 ml) and water (2 × 10 ml), dried with anhydrous magnesium sulphate and the solvent was removed by rotary evaporation. The remaining semi--solid was chromatographed on a 3 × 30 cm column of 20—100 μm silica gel deactivated with 15% of water using light petroleum–toluene–acetone (5 : 5 : 1) as eluant. The collected fractions were analyzed by TLC using the same solvents (4 : 4 : 1). A mixture of two compounds (67 mg, fraction A) with R_F 0·47 and 0·41 was eluted first, followed by the unreacted *I* (20 mg, R_F 0·32) and a fraction showing a single spot with R_F 0·17 (35 mg, fraction B). HPLC analysis showed

that the fraction B consisted of two components which were separated by preparative HPLC yielding *III* (24 mg, 18%) and *IV* (11 mg, 4·5%).

N-*Butyl*-4-*hydroxy*-3-*methoxy*-6-*nitrobenzamide* (III), m.p. 188—191°C. For $C_{12}H_{16}N_2O_5$ (268·3) calculated: 53·72% C, 6·02% H, 10·44% N; found: 53·65% C, 6·03% H, 10·49% N. High-resolution mass spectrum: calculated 268·1059; found 268·1065. ^{1}H NMR spectrum: 7·67 (bs, H-5), 6·91 (bs, H-2), 4·01 (s, OCH_3), 5·66 (bt, NH, $J = 6·8, 6·8$), 3·45 (q, CH_2N, $J = 6·8, 6·8$, 6·8), 1·40 (m, CH_2), 1·60 (m, CH_2), 0·97 (t, CH_3). UV spectrum λ_{max}, nm (ε, m^2 mol^{-1}): 341 (560), 298 (533), 242 (1 490); UV spectrum of the corresponding anion generated in 50% aqueous methanol, 0·01M-NaOH: λ_{max}, nm (ε, m^2 mol^{-1}): 408 (342), 318 sh (450), 268 (2 238).

6,6′-*Bis*(N-*butylcarbamoyl*)-3,3′,4,4′-*tetramethoxyazoxybenzene* (IV), m.p. 182—184°C. For $C_{31}H_{36}N_2O_5$ (516·7) calculated: 63·35% C, 7·04% H, 5·42% N; found: 63·28% C, 7·09% H, 5·13% N. High-resolution mass spectrum: calculated 516·2624, found 516·2640. ^{1}H NMR spectrum: 7·28 (bs, H-2′), 7·78 (bs, H-5′), 8·30 (bs, H-2), 7·14 (bs, H-5), 3·95 (s, OCH_3), 3·96 (s, OCH_3), 3·98 (s, OCH_3), 4·00 (s, OCH_3), 6·19 (bt, NH, $J = 5·5, 5·5$), 3·30 (dt, CH_2N, $J = 5·6$, 7·0, 7·0), 3·37 (dt, CH_2N, $J = 5·6, 7·0, 7·0$), 1·17 (m, CH_2), 1·39 (m, CH_2), 0·77 (t, CH_3, $J = 7·0, 7·0$). UV spectrum λ_{max}, nm (ε, m^2 mol^{-1}): 364 (525), 261 (2 365). IR spectrum: 1 661 (C=O), 3 438, 3 365 (N—H), 1 278 (C—O).

Fraction A was subjected to flash-chromatography on a water-jacketed ($13 \pm 2°C$) $50 \times \times 2·5$ cm column of 30—60 μm silica gel deactivated with 10% of water. Elution with light petroleum–toluene–acetone (10 : 10 : 1) gave *IIb* (23 mg, 18%) and *IIa* (44 mg, 32%) as yellow oils of HPLC purity greater than 99%.

N-*Butyl*-4-(N-*butylamino*)-3-*methoxy*-6-*nitrobenzamide* (IIa). For $C_{16}H_{25}N_3O_4$ (323·4) calculated: 59·41% C, 7·81% H, 12·99% N; found: 59·48% C, 7·82% H, 12·85% N. High-resolution mass spectrum: calculated 323·1845; found 323·1854. ^{1}H NMR spectrum: 7·17 (bs, H-5), 6·76 (bs, H-2), 3·94 (s, OCH_3), 5·00 (bt, NH, $J = 5·5, 5·5$), 5·54 (bt, NH, $J = 5·8, 5·8$), 3·18 (dt, CH_2N, $J = 5·5, 7·0, 7·0$), 3·43 (dt, CH_2N, $J = 5·8, 7·0, 7·0$), 1·42 (m, CH_2), 1·62 (m, CH_2), 0·97 (t, CH_3, $J = 7·0, 7·0$), 0·98 (t, CH_3, $J = 7·0, 7·0$). UV spectrum λ_{max}, nm (ε, m^2 mol^{-1}): 394 (127), 298 sh (355), 264 (1 149).

N-*Butyl*-3-(N-*butylamino*)-4-*methoxy*-6-*nitrobenzamide* (IIb). For $C_{16}H_{25}N_3O_4$ (323·4) calculated: 59·41% C, 7·81% H, 12·99% N; found: 59·35% C, 7·82% H, 12·88% N. High-resolution mass spectrum: calculated 323·1845; found 323·1855. ^{1}H NMR spectrum: 7·55 (bs, H-5), 6·44 (bs, H-2), 3·93 (s, OCH_3), 5·56 (bt, NH, $J = 6·0, 6·0$), 5·03 (bt, NH, $J = 5·8, 5·8$), 3·22 (dt, CH_2N, $J = 5·8, 7·0, 7·0$), 3·47 (dt, CH_2N, $J = 6·0, 7·0, 7·0$), 1·40 (m, CH_2), 1·60 (m, CH_2), 0·96 (t, CH_3, $J = 7·0, 7·0$), 0·97 (t, CH_3, $J = 7·0, 7·0$). UV spectrum: λ_{max}, nm (ε, m^2 mol^{-1}): 401 (543), 268 (380), 243 (399).

Determination of the Relative Rate of Photolysis

Either *I* (27·8 mg; 0·099 mmol) or *V* (18·5 mg; 0·101 mmol) were dissolved in 25 ml of hot tert--butanol and the cooled solution was mixed with borate buffer (0·1 moll^{-1}, pH 11·0, 25 ml). Butylamine (150 μl; 1·5 mmol) was added, the mixture was deoxygenated with a stream of argon (30 min) and irradiated in a cylindrical magnetically stirred quartz photoreactor using external light source, an Osram HBO-200 mercury lamp equipped with a glass lens and water filter (path length 10 cm). Samples (70 μl) were taken off and, after dilution with methanol to 2·00 ml, their UV spectra were measured. The substrate actual-to-initial concentration ratio was calculated using the expression $c/c_0 = (A_\infty - A)/(A_\infty - A_0)$, where A_0, A, and A_∞ stand for the initial,

actual, and infinite-time absorbance at 265 nm (compound *I*) or 267 nm (compound *V*). The values of A_∞ were obtained by means of graphical extrapolation.

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Translated by the author (P.K.).