ALLOXAN-(*o*-AMINO-ANIL) AS MODEL CHROMOPHORE FOR A 10,10a-RING OPENED INTERMEDIATE IN FLAVIN-DEPENDENT DIOXYGEN ACTIVATION

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1. Introduction

The mechanism of flavin-mediated dioxygen activation may involve reversible opening of the flavin ring system, as proposed [1] for aromatic hydroxylases and [2] for bacterial luciferase. A 4a,5-open intermediate has been considered as a possible candidate for a transient with λ_{max} = 390–420 nm (ϵ = 12 000–15 000 M^{-1} cm^{-1}) detected in reactions of *p*-hydroxybenzoate hydroxylase [3]. Ring-opening between positions 4a,5 has been questioned [4] in favor of rearrangement and alternative bond cleavage, now proposed to involve positions 10,10a, which gives rise to the hypothetical structures IVa and IV (scheme 1) [5].

The spectral properties of chromophores II and IV (scheme 1) have been long subject to speculation. Blue transients were observed in two synthetic routes to flavins [6,7], for which structure IV was assumed by analogy with the long known alloxan-*p*-dimethylaminoanil [8]. Analogy with that model compound has also been the justification for the proposal [4,9], that, 1,10a-dihydroflavin II (1-RFl-10a-X, scheme 1) might be a blue chromophore. The corresponding assumption, that ring closure IV $\longrightarrow$ II would not essentially alter the π-electron system, turned out to be wrong [10,11]. We later obtained a crystalline, stable, yellow 1,10a-dihydroflavin II (R^{10}–R^1=CH_2–CH_2, R^3=CH_3, R^7=R^8=H, X=OCH_3) with λ_{max} = 404 nm (ϵ = 4300 M^{-1} cm^{-1}) [11], which is reversibly formed from the corresponding cation III (scheme 1) by very cautious stoichiometric addition of strong nonaqueous base. This newly isolated compound differs from the general structure II only by the 1,10-ethano bridge, which renders the skeleton more rigid.

We now report the preparation of a stable blue specimen of structure IV, and we compare it with the blue autooxidation product of 1,5-dihydro-1,3-dimethyllumiflavin (I, R^1=R^3=R^7=R^8=R^{10}=CH_3; 1-RFl_{red}H, scheme 1) [4,12,13].

2. Materials and methods

2.1. *Synthesis of 2′-(*t*-butylamino)-1,3,4′,5′-tetramethylalloxan-5-anil (IV; R^1=R^3=R^7=R^8=CH_3, R^{10}-*t*-butyl)*

1-*t*-Butylamino-2-nitro-4,5-dimethylbenzene (1 mmol; obtained from 1,2-dinitro-4,5-dimethylbenzene (2 g) and *t*-butylamine (20 ml) by heating 24 h at 130°C, sealed tube, yield 60% after recrystallization from ethanol) in methanol was reduced with H_2/Pd. After evaporation of methanol, the dry residue was dissolved in acetonitrile and added to tetramethylalloxantine [14] (1 mmol) suspended in dry acetonitrile (70 ml) at 60°C in a dry reflux apparatus. The solution rapidly turned blue and was stirred for several hours. The blue product ran as a single spot on thin-layer chromatography. After evaporation of the solvent, the blue compound was crystallized from hexane with a yield of 39%, m.p. 149–151°C.

$C_{18}H_{24}N_4O_3$ (344,4): Calc., C 62.77, H 6.98, N 16.27; found, C 62.61, H 7.12, N 15.98.
IR(KBR): 1700, 1640 (C(2,4,6)=0) cm^{-1}.
UV (Acetonitrile): λ_{max} (log ϵ) = 350 (3.95), 620 (4.05) nm.

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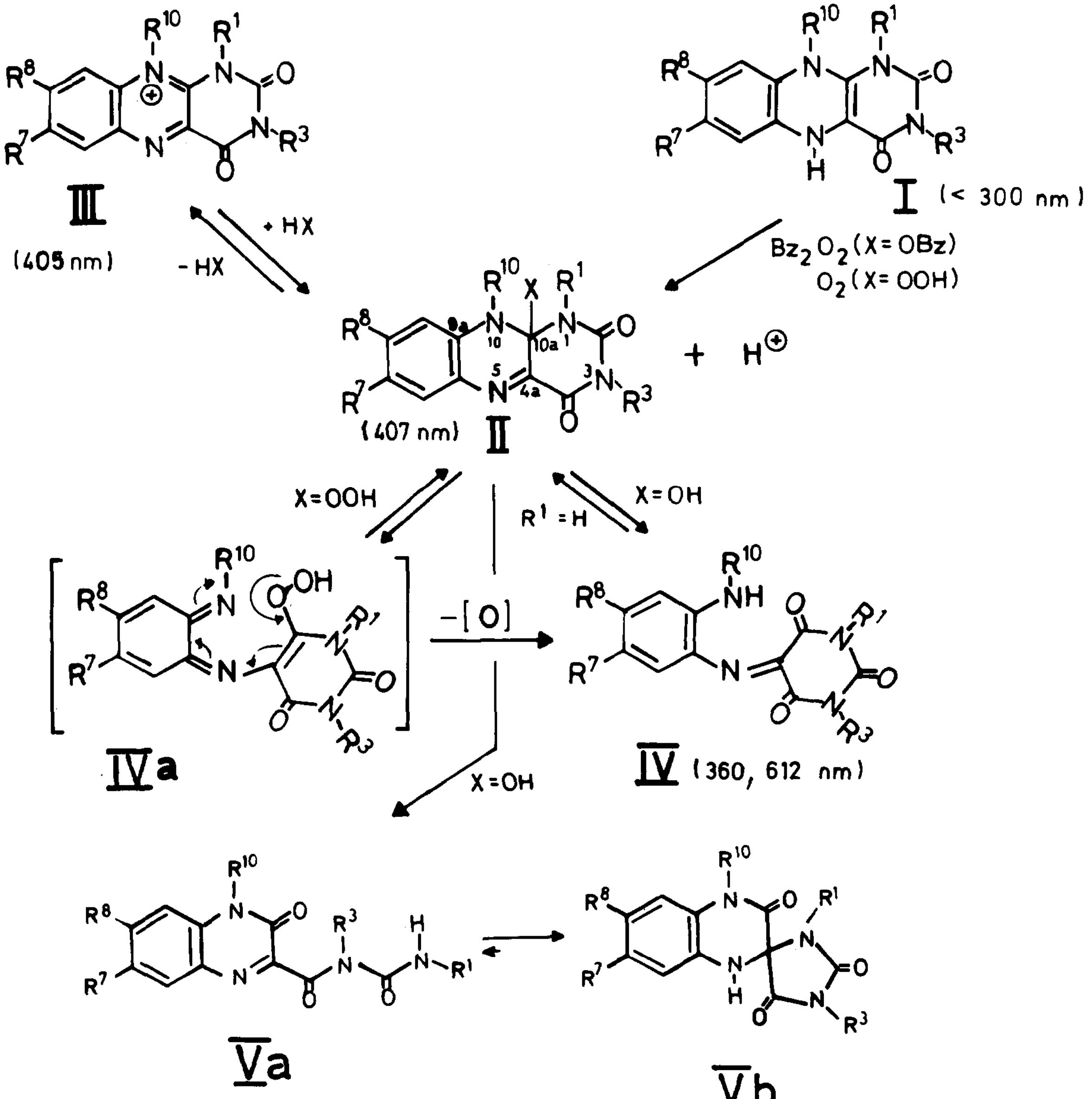
^{1}H NMR ($CDCl_3$): 8.03 (s; 1 H, Ar-H), 6.70 (s; 1 H, Ar-H), 3.43 (s; 6 H, N(1,3)-CH_3), 2.25 (s; 3 H, Ar-CH_3), 2.12 (s; 3 H, Ar-CH_3), 1.49 (s; 9 H, *t*-butyl).

MS (70 eV): 344 (100%, M^+)

2.2. *Oxidation of 1,5-dihydro-1,3-dimethyl-lumiflavin [15] (I; R^1=R^3=R^7=R^8=R^{10}=CH_3)*

Dibenzoyl peroxide ($(BzO)_2$) was recrystallized from chloroform/methanol and was 98–99% pure by iodometric titration. Acetonitrile was purified to spectroscopic standards, benzene and xylene (isomeric mixture, Merck) were either dried over molecular sieves or saturated with water at room temperature. Stock solutions of dibenzoyl peroxide (2–3 mM) were prepared daily from weighed amounts of reagent and deoxygenated solvent and stored under argon. All solutions were purged with argon for ≥1 h to ensure removal of oxygen. Anaerobic experiments were carried out in a Thunberg type cuvette containing



Scheme 1

5–8 × 10^{-5} M dihydroflavin and 3 × 10^{-4} M benzoic acid in either benzene or xylene with a calculated amount of dibenzoyl peroxide. The dihydroflavin I was standardized by oxidation to the 1,3,10-trimethylflavinium cation (III; R^1=R^3=R^7=R^8=R^{10}=CH_3) by first adding trifluoroacetic acid (20 μl for a cuvette volume of 3 ml) and then a slight excess of benzoyl peroxide. ϵ_{max} (400 nm) was then compared with that of an authentic sample of the cation III [15], dissolved in acetonitrile and then diluted 1:60 with xylene or benzene (ϵ^{400}_{max} = 16 400 (benzene), 14 100 (xylene)).

3. Results and discussion

3.1. *Synthesis of the blue chromophore IV (scheme 1)*

To suppress 1,10a-dehydration following 10,10a-ring closure in chromophore IV, N(1,3) had to be blocked by methyl groups (R^1=R^3=CH_3). Furthermore, we had to introduce a *t*-butyl residue in position N(10) (R^{10}=*t*-butyl), since primary and secondary alkyl and unsubstituted aryl groups are labile [7]. These authors have already reported on the occurrence of a blue intermediate (considered to be an anil IV; R^1=R^3=R^7=R^8=H, R^{10}=mesityl), but could not characterize their compound further.

In fig.1 we compare the optical spectrum of the blue oxidation product (a) of 1,5-dihydro-1,3-10-trimethylflavin (I; R^1=R^3=R^7=R^8=R^{10}=CH_3, 1-$RFl_{red}H$), first observed in 1960 [17], with *N,N*-dimethyl-alloxan-*o*-(*t*-butylamino-)anil IV (R^1=R^3=R^7=R^8=CH_3, R^{10}=*t*-butyl (b) obtained now. Both chromophores have A_{max} at 360 and 600 nm, which differ by < ± 10 nm. The extinction coefficients in

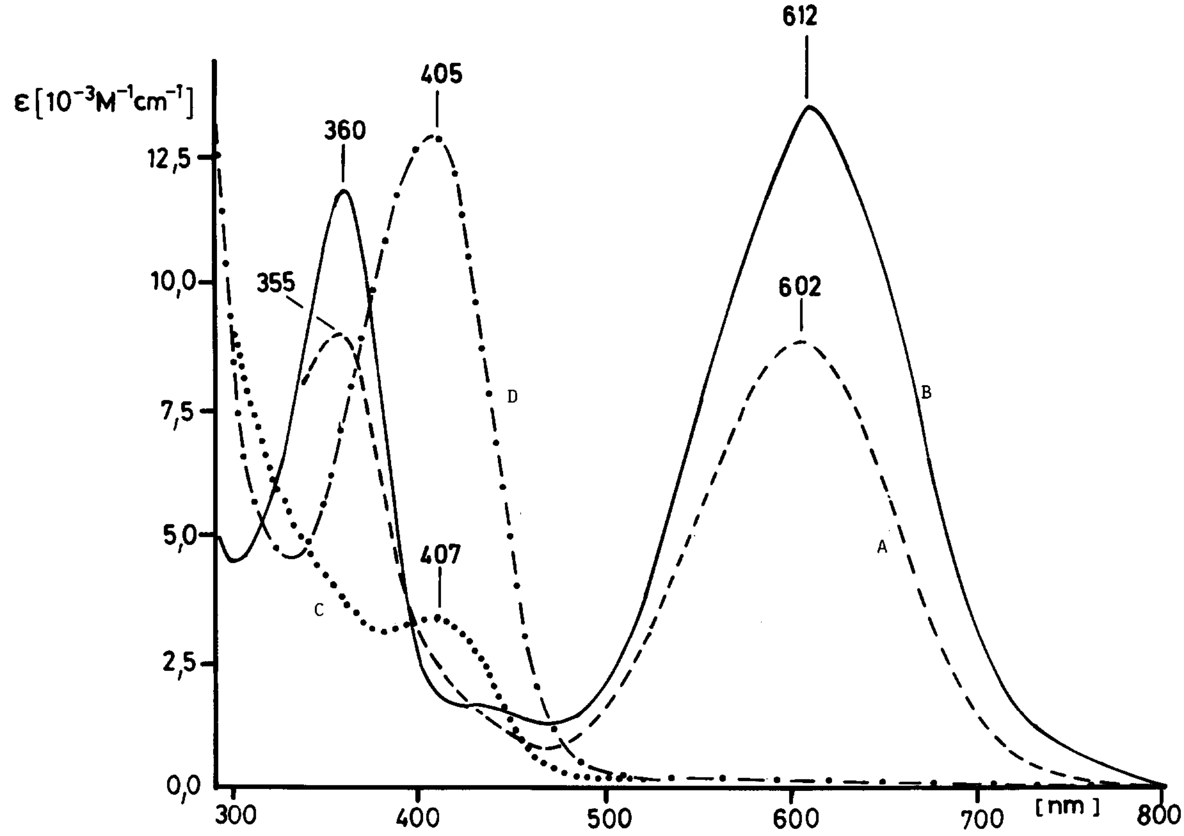


Fig.1. (a) Spectrum obtained after oxidation of I (R^1=R^3=R^7=R^8=R^{10}=CH_3) under optimum conditions. Xylene saturated with water and air; final concentration of 1.5-dihydro-1,3,10-trimethylflavin, 5–8 × 10^{-5} M; 3 × 10^{-3} M benzoic acid and 1 mol dibenzoyl peroxide/mol flavin. Under these conditions the decay of the chromophore is relatively slow ($t_{1/2}$ ~4 h). (b) (——) IV (R^1=R^3=R^8=CH_3, R^{10}=*t*-butyl). (c) (· · ·) Spectrum obtained after oxidation of I (R^1=R^3=R^7=R^8=R^{10}=CH_3) under conditions, as in (a) but without oxygen (=II). (d) ('—·—) (c) after addition of 20 μl CF_3CO_2 H (=III).

xylene for the *t*-butyl anil are $\epsilon_{\max}^{360} = 11\,600\ \mathrm{M^{-1}\,cm^{-1}}$ and $\epsilon_{\max}^{612} = 13\,000\ \mathrm{M^{-1}\,cm^{-1}}$, compared with values of $\epsilon_{\max}^{355} = 8750\ \mathrm{M^{-1}\,cm^{-1}}$ and $\epsilon_{\max}^{602} = 8930\ \mathrm{M^{-1}\,cm^{-1}}$ for the blue product, obtained by oxidation of 1-$\mathrm{RFl_{red}H}$.

3.2. *Oxidation of 1,5-dihydro-1,3-dimethyl-lumiflavin (I, 1-RFl$_{red}$H; R^1=R^3=R^7=R^8=R^{10}=CH_3; scheme 1)*

The blue oxidation product of 1-$\mathrm{RFl_{red}H}$, inferred above to be an anil IV ($\mathrm{R^1{=}R^3{=}R^7{=}R^8{=}R^{10}{=}CH_3}$) by comparison with the synthesized homologue was obtained with optimum yield using as oxidants dioxygen and dibenzoylperoxide $\mathrm{(BzO)_2}$ in water-saturated xylene. Oxidation of 1-$\mathrm{RFl_{red}H}$ by dioxygen alone (1) is a very slow reaction, taking several hours for completion in apolar solvent, yielding only small amounts of the anil IV, the main product being the spirohydantoin Vb (scheme 1; $\mathrm{R^1{=}R^3{=}R^7{=}R^8{=}R^{10}{=}CH_3}$) probably formed by 1,10a-ring opening of the intermediate 1-RFl-10a-OH (II; $\mathrm{R^1{=}R^3{=}R^7{=}R^8{=}R^{10}{=}CH_3}$, X=OH) via the corresponding carboxyureide Va [15].

4. Conclusions

The hitherto-known types of enzyme bound flavin–oxygen adducts have the chromophore 5-HFl-4a-X: Transient I (X=OOH) and transient III (X=OH) observed during action of *p*-hydroxybenzoate hydroxylase [3] and complex II (X=OOH) observed with bacterial luciferase [16]. For the intermediate transient II in the oxygenative action of *p*-hydroxybenzoate hydroxylase, we have proposed [11] the chromophore 1-$\mathrm{HFl_{ox}^{+}}$ (scheme 1; III; $\mathrm{R^1{=}R^3{=}H}$, $\mathrm{R^7{=}R^8{=}CH_3}$, $\mathrm{R^{10}}$=Rib.). The reaction sequence would thus be:

$$\text{5-HFl}_{\text{red}}\text{H} \xrightarrow{+\mathrm{O_2}} \text{5-HFl-4a-OOH} \xrightarrow[\text{A}]{-[\mathrm{O}],\,-\mathrm{H_2O}} \text{1-HFl}^{+}_{\text{ox}} \xrightarrow[\text{B}]{+\mathrm{H_2O}}$$

$$\text{5-HFl-4a-OH} \xrightarrow{-\mathrm{H_2O}} \text{Fl}_{\text{ox}}$$

agreeing with the concept [17], which postulates the occurrence of rhythmic prototropic shifts between the flavin positions 1 and 5, induced by alternating conformations of the apoprotein. Apparently, the cleavage of the O–O bond requires a 1-protonated intermediate flavin species, while the preceding 4a-hydroperoxide is only a 'dioxygen store'. Thus, reaction A must consist of at least 4 substeps: Rearrangement to the active 1-HFl-10a-OOH (formula II, X=OOH), 10,10a-cleavage to yield a very unstable vinylogous peracid-amide (IVa, scheme 1), which 'shoots' an oxene unit upon the substrate to yield IV. The latter undergoes 10,10a-reclosure to yield 1-HFl-10a-OH, which in turn rapidly splits $\mathrm{OH^-}$ to form 1-$\mathrm{HFl_{ox}^{+}}$. By studies of alkylated models all chromophores involved in this scheme are meanwhile known: The clue intermediate is IV which is, chemically, extremely sensitive and may still be very shortlived biologically, but is easily detectable at 600 nm.

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