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Structural factors influencing the reaction rates of 4-aryloxy-7-nitrobenzofurazans with amino acids

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Abstract: An interesting observation was made when studying the S_N Ar reaction between several 4-aryloxy-7-nitrobenzofurazans (2) and several amino acids leading to the apparition of detectable fluorescence from the substitution products 3. Acidic amino acids reacted very slowly=while basic amino acids react fastest with 2 having an unsubstituted phenyl or a 4-formyl-phenyl Ar group. Amongst neutral amino acids, proline reacts fastest at room temperature after 100 min. with 2 having a methoxy-substituted Ar group. (c) Central European Science Journals. All rights reserved.

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1 Introduction

During the exploration of potential antileukemic drugs, it was observed [1–3] that 4chloro-7-nitrobenzofurazan (4-nitrobenzo-2-oxa-1,3-diazole, NBD) **1**, a nonfluorescent compound, reacted with amino acids, primary or secondary amines affording detectably fluorescent substitution products, **3**. Analytical applications of these compounds followed soon [4–43].



Scheme 1

In previous communications we examined the reaction of NBD chloride (1) with various phenols, which led to the synthesis of several 4-aryloxy-7-nitrobenzofurazans (2) that are nonfluorescent or only weakly fluorescent [44–46]. In the present paper we report a qualitative kinetic study of six such compounds (2a - 2f) with three pairs of amino acids (AAs): two acidic AAs (Asp and Glu), two neutral AAs (Val and Pro), and two basic AAs (Lys and Arg). All the amino acids were chosen from the the naturally occuring =L series. The compound 2f was obtained by substituting the chlorine atom of 1 with the cytostatic Etoposide [47].

2 Results

The reaction of **2a** with some amino acids was reported to yield fluorescent products **3** [48]. We decided to investigate the reaction of **2a**–**2f** with six representative amino acids in order to study the influence of the aryl group Ar in **2**.

On reacting the six amino acids from the three classes having markedly different isoelectric points (pI) with six 4-aryloxy-7-nitrobenzofurazans (2a–2f), it was observed that the intensity of the fluorescence varied in time with differing rates. Trends observed are shown in Figures 1a – 1f. Although the isoelectric point in Table 1 is for aqueous solutions and the solvent used in our studies is 99.6 % ethanol, the order of pI values will not vary considerably. The acidic amino acids and the neutral amino acid with a primary amino group (Val) produced practically a constant intensity, whereas the other three amino acids led to a increasing intensity. At 25 °C and after 100 minutes, the intensity of fluorescence (in arbitrary units) is presented in Table 1; concentrations of the reactants in 99.6 % ethanol were 2×10^{-3} mol/L for 2a-2f, and 4×10^{-3} mol/L for the amino acid. The data are averages of five experiments (error ± 0.3 %).

| Class of AA | Amino Acid | $\mathbf{pI}~^{49}$ | 2 a | 2b | 2 c | 2 d | 2e | 2f |
|-------------|---------------|---------------------|------------|------------|------------|------------|-----|------------|
| Acidic AAs | Aspartic Acid | 2.77 | 79 | 173 | 105 | 100 | 76 | 68 |
| | Glutamic Acid | 3.22 | 81 | 211 | 14 | 101 | 45 | 66 |
| Neutral AAs | Valine | 5.96 | 92 | 88 | 98 | 114 | 81 | 72 |
| | Proline | 6.30 | 117 | 631 | 1320 | 121 | 299 | 589 |
| Basic AAs | Lysine | 9.74 | 151 | 369 | 403 | 243 | 717 | 443 |
| | Arginine | 10.76 | 180 | 278 | 322 | 160 | 378 | 321 |

Table 1 Intensity of the fluorescence of the reaction products (in conditions mentioned in Figure 1) between the six amino acids and the six compounds $2\mathbf{a}-2\mathbf{f}$ ($\lambda_{ex}=480$ nm, $\lambda_{em}=540$ nm), arbitrary units (a.u.).

The highest reaction rate (denoted by **boldface** characters in Table 1) was between one of the two basic amino acids and compounds 2 having an unsubstituted (2a) or a 4-formyl-phenyl Ar group (2d, 2e), whereas proline reacted fastest with compounds 2 having electron-donating methoxy groups (2b, 2c, 2f).

A caveat is necessary before discussing the results. Two detailed measurements were carried out in 99.6 % ethanol for absorption (at about 0.5 mmol/L concentration) and emission spectra (at 0.7 mmol/L concentration) of the reaction products of NBD with the two neutral amino acids: **5** (with valine), and **6** (with proline). The synthesis of **5** and **6** is described in the Experimental Part. Absorption bands for **5** are at $\lambda_{max} = 334$ nm ($\varepsilon = 240 \text{ L/mol}\times\text{cm}$) and 459 nm ($\varepsilon = 600 \text{ L/mol}\times\text{cm}$); for **6** at 347 nm ($\varepsilon = 1,100$ L/mol×cm) and 486 nm ($\varepsilon = 3,400 \text{ L/mol}\times\text{cm}$). Quantum yields (Φ_f) were measured using quinine sulfate in 0.1 N sulfuric acid as standard with $\lambda_{ex} = 355$ nm ($\Phi_f = 0.55$). The emission band with $\lambda_{ex} = 430$ nm for **5** is at 536 nm, I_f = 246 a.u., $\Phi_f = 0.022$; for **6** at 541 nm, I_f = 541 a.u., $\Phi_f = 0.009$. These determinations show that even taking into account the different quantum yields (less that 1 : 2.5) there is a significant difference



between reaction rates, which will be discussed in the next Section.

Fig. 1 Variation in time of the intensity of fluorescence (If) during the reaction (in 99.6 % ethanol at 25 °C) of amino acids with compounds 2a–2f (λ_{ex} = 480 nm, λ_{em} = 540 nm); concentration 2a-f = 2×10⁻³ mol/L; concentration of amino acid = 4×10⁻³ mol/L.

3 Discussion of the results

In order to explain the above observation about reaction rates, one must take into account that (i) the benzofurazan ring has a stronger inductive effect and a lower resonance effect than a nitro group [50]; (ii) the combined result for the nitrobenzofurazan is a strong electron-withdrawing effect facilitating the S_NAr substitution; (iii) methoxy and aryloxy substituents appear to be better leaving groups than chloro; and (iv) there is a transition state leading to the immediate formation of a red-colored Meisenheimer complex 4 [51– 55] and then, more slowly, to the substitution product 3 that is detectably fluorescent. One may assume that the basic AAs, which are more nucleophilic than all other AAs, prevail whenever the aryloxy group is not activated (2a) or deactivated by an electronwithdrawing formyl group (2d), even when there are methoxy groups (2e).

The structure of the Meisenheimer complex (4) is presented in Scheme 2. It may either regenerate the reactants (2 and the amino acid), or it may afford the reaction products, the phenol and the fluorescent compound 3. In the particular case of two amino acids, the products 3 are shown separately under the designation 5 and 6.



Scheme 2

The products 5 and 6 obtained from all compounds 2a-2f and value or proline, respectively, are depicted in Scheme 3.

A plausible explanation for the unexpected observation that proline, a neutral amino acid, reacts fastest with the 4-aryloxy-7-nitrobenzofurazans 2 that have one or two electron-donating methoxy groups and no electron-withdrawing groupscan be obtained by asserting a parallelism with the Brønsted linear free energy relationship for acid-base catalysis which states that the logarithm of the reaction rate is proportional to pK_a^{α} or pK_b^{β} . When the exponent α or β is close to zero, no catalysis is observed because the protic solvent assumes the role of catalyst; when the exponent is close to 1, the strongest acid or base (the lyonium/lyate ion) prevails and specific catalysis results; and when the exponent is around 0.5, one observes general acid-base catalysis because the medium-



Scheme 3

nucleophilicity (or electrophilicity) prevails. Possibly the fact that our experiment was carried out in a solvent that has only 0.4 % of water in ethanol may also influence the outcome. Then to answer the question of the faster reaction of proline, which is also a neutral amino acid we should consider that among all amino acids, proline is the only one with a more basic secondary amino group, and this fact is reflected in the higher pI (Table 1) of Pro than for Val. Keeping in mind that a difference of 0.3 logarithmic units is equivalent to half the value of the hydronium ion concentration for Pro at the pI. One must also consider that the stability of reaction products **3a–3f** differs; indeed, when they are derived from primary amino groups (e. g. **5**) their stability must be lower than when they are derived from Pro (product **6**). To be more specific, in the resonance formulae of products **5** and **6** having a negative charge on the nitro group and a positive charge on the nitrogen of the amino acid, this latter "ammonium" nitrogen is better stabilized in **6** with N bonded only to carbon atoms than in **5** which has an N–H bond.

The previous discussion concerned comparisons of columns in Table 1, marking in each case one fastest reaction in **boldface** characters. In these comparisons the reaction products (and their fluorescence intensities) differ, therefore the comparisons are mainly qualitative, considering also the fact that Φ_f values for **3** differ. Now comparing the data in the rows in Table 1 corresponding to a single amino acid and resulting in the same product **3**, one can see that Asp reacts with all six 4-aryloxy-7-nitrobenzofurazans (**2a**– **2f**) with rates that differ only slightly, between 76 and 173 (in arbitrary units). So does also Val (from 72 to 115), and to a certain extent Arg when the intensity ranges from 160 to 378, i. e. approximately from simple to double, but with a considerably higher rate. By contrast, the variation range is larger for the remaining AAs: for Glu from 14 to 211 (a factor of 15), for Pro from 117 to 1320 (a factor of 11), and for Lys from 151 to 717 (a factor of about 5). A general conclusion is that the three AAs (Asp, Glu, Val) have lower reaction rates than the last three AAs (Pro, Lys, Arg) that are more nucleophilic as attested by their pI values.

In the Experimental Section, the synthesis of substitution products 5 and 6 with Val and Pro and their characterization by elemental analysis, ¹H-NMR and ¹³C-NMR spectra are detailed. Attempts to obtain the analogous product with Lys led to reaction mixtures derived by reaction with either the α -amino or the ε -amino group and no disubstitution product was detected.

The NMR spectra of compound 5 in CD_3OD presents a broadened peak for H-5 from the C-4–NH bond, with a partial double bond character, leading to restricted rotation. The signal due to H-3' is an octet because the isopropyl methyl groups, H-4', are diastereotopic while the signal due to H-2' is coupled to NH/ND and like the COOH proton, cannot be seen in CD_3OD . Also, broadened signals are observed in ¹³C-NMR for COOH, CH-5, and C-2' probably due to the afore-mentioned slow rotation.

In the ¹H-NMR spectrum of compound **6** at 50 °C the H-5 proton appears as a sharp doublet at $\delta = 6.28$ ppm with J = 8.8 Hz because at this temperature the rotation is fast at the NMR time scale. Otherwise, the same remarks hold true as for **5**, including the similarity of chemical shifts in ¹³C-NMR for COOH, CH-5, and C-2'.

Our observations on the fairly rapid reaction of compounds 2 with amino acids (and certainly also with polypeptides) have the following important consequences: (i) compounds 2 can have biological effects; (ii) they can be used to tag by fluorescence amino acids, polypeptides and proteins; (iii) they may be also be applied in analytical determinations.

4 Conclusion

On investigating the reaction between six 4-aryloxy-7-nitrobenzofurazans (2a–2f) and six amino acids leading to the observation of detectable fluorescence due to the substitution products, it was observed that (i) the basic amino acids react fastest with compounds 2a, 2c and 2d having a strong electron deficient group at the aromatic carbon where the S_N Ar reaction takes place, and that (ii) proline reacts fastest with compounds 2a, 2b and 2f having electron-donating methoxy groups. This observation can be rationalized by assuming that in the former case the strongest nucleophiles prevail, while in the latter case the secondary amino group of proline is the "winner in the race" because it leads to a more stable product 3. A possible additional structural feature responsible for the observed effect is the difference in the steric environment of the nucleophilic amino group of the amino acid leading to overcrowding in the intermediate Meisenheimer complex 4; further studies will be necessary in order to check this hypothesis.

5 Experimental part

The synthesis of compounds 2a-2f was described in previous papers [44–46]. For TLC separations Merck silica gel plates GF_{254} were used. 4-Chloro-7-nitrobenzofurazan (1) was purchased from Merck, and amino acids were commercial products and used without further purification. ¹H-NMR and ¹³C-NMR spectra were recorded with a Varian Gemini 300BB spectrometer (300 MHz for ¹H and 75 MHz for ¹³C). For fluorescence spectra a Perkin-Elmer 204 spectrofluorimeter was used; conditions are specified in Table 1 and Fig. 1. For fluorescence, an excitation lamp (Xe, 150 W) interfaced with the computer was used, allowing a pre-established data reading time of 0.5 s.

Synthesis of compounds 5 and 6. Although the formulae of compound 5 and of the analogous compound involving the ε -amino group of lysine are mentioned in the literature [56–62], these compounds do not appear to have been obtained and characterized.

According to literature data,⁴² a solution of the L-amino acid (valine or proline, 1 g in 16 mL of water) and sodium hydrogen carbonate (molar ratio 1:3 amino acid:NaHCO₃) was treated with 1 g of **1** in 40 mL of methanol. The reddish-brown fluorescent solution was kept at 55 °C for 1 h with stirring in the case of valine, or only for 5 minutes for proline. Then the pH was adjusted to 1.5 with 1M hydrochloric acid, followed by extraction with methylene chloride. The extract was dried overanhyd. Na₂SO₄, concentrated under vacuum, and separated by preparative TLC on silica gel using CH₂Cl₂:AcOH 9:1 v/v. The main colored zone ($R_f = 0.56$) contained compound **5**, which was isolated in a Soxhlet extractor with a mixture 7:3 v/v of CH₂Cl₂:MeOH. Evaporation of the solvent under vacuum gave **5** as a pure crystalline compound. Compound **6** crystallized from the HCl-acidified solution on cooling, was isolated by filtration, and purified as indicated above by TLC.

1'-(7-Nitro-4-benzofurazanyl)valine (5), yield 74 %, m.p. 134-136 °C (in open capillary); Anal.: calcd. for $C_{11}H_{12}N_4O_5$: C 47.15; H 4.32; N 19.9; found C 47.13, H 4.31, N 19.96 %.

¹H-NMR (CD₃OD, δ ppm, J Hz): 8.50 (d, 1H, H-6); 6.42 (bd, 1H, H-5, J = 8.9); 4.30 (vls, 1H, H-2'; J = 8.9); 2.43 (octet, 1H, H-3', J = 6.8); 1.16 (d, 3H, CH₃-4', J = 6.8); 1.12 (d, 3H, CH₃-4', J = 6.8).

¹³C-NMR (CD₃OD, δ ppm): 174.96 (COOH); 145.81 (Cq); 145.47 (Cq); 121.10 (Cq); 138.07 (CH-6); 101.34 (CH-5); 64.89 (C-2'); 32.30 (C-3'); 19.51 (C-4'); 19.22 (C-4').

1'-(7-Nitro-4-benzofurazanyl)proline (6), yield 60 %, m.p. 155-156 °C (in open capillary), lit. m.p. 156-157 °C;⁵ Anal.: calcd. for $C_{11}H_{10}N_4O_5$: C 47.49; H 3.62; N 20.1; found C 47.47; H 3.60; N 20.10 %.

¹H-NMR (CDCl₃ with 10% DMSO-d₆, δ ppm, J Hz): 8.57 (d, 1H, H-6, J = 9.0); 6.28 (bs, 1H, H-5); 3.95 (bs, 1H, H-2'); 3.42 (m, 2H, H-5'); 2.43 (m, 1H, H-3'); 2.08 (m, 3H, H-3'+ 2 H-4').

¹³C-NMR (CDCl₃ with 10 % DMSO-d₆, δ ppm): 177.22 (COOH); 144.96 (Cq); 144.24

(Cq); 143.86 (Cq); 137.35 (CH-6); 122.09 (Cq); 103.14 (CH-5); 64.26 (C-2'); 50.92 (C-5'); 31.12 (CH_2) ; 22.72 (CH_2) .

Compound **6** was reported [43] to have m.p. 156-157 °C, and to present fluorescence in acetonitrile with $\lambda_{em} = 535$ nm ($\lambda_{ex} = 469$ nm).

Reaction of 1 with lysine. A similar reaction between compound 1 and lysine (molar ratio 1:1 Lys:1) led to a mixture of ε -(7-nitro-4-benzofurazanyl)lysine and α -(7-nitro-4benzofurazanyl)lysine in comparable amounts as indicated by ¹H-NMR spectra, which revealed two signals for H-5, H-6, and H-6' (the last for the ε -CH₂ group). Alternatively, a CD₃OD solution of lysine and 1 in molar ratio 1:1 was introduced into the NMR vial, and spectra were recorded at various times till 24 h.

¹H-NMR (CD₃OD, δ ppm, J Hz): 8.51 (d, $\frac{1}{2}$ H, H-6, J = 8.9); 8.45 (d, $\frac{1}{2}$ H, H-6, J = 8.9); 6.37 (d, $\frac{1}{2}$ H, H-5, J = 8.9); 6.29 (d, $\frac{1}{2}$ H, H-5, J = 8.9); 4.21 (bs, $\frac{1}{2}$ H, H-6'); 4.03 (bs, $\frac{1}{2}$ H, H-6'); 3.51(m, H-2'); 2.15-1.43 (m, 6H, H-3'-4'-5') (in the above data, the mention $\frac{1}{2}$ H refers to the approximate integral area of the signal).

¹³C-NMR (CD₃OD, δ ppm): 174.26 (COOH); 145.78 (Cq); 144.89 (Cq); 140.62 (Cq); 133.59 (C-6); 112.34 (Cq); 103.64 (C-5); 55.72 (C-6'); 40.30 (C-2'); 31.53 (CH₂); 28.09 (CH₂); 23.08 (CH₂-4').

TLC Separations. The reaction between amino acids and compounds 2a-2f was monitored using the synthesized products 5 and 6, as well as the resulting phenols (ArOH a-f) as standards on silica gel plates GF₂₅₄. Mobile phases were: CH₂Cl₂:AcOH 9:1 (v/v) for 4 and 5; for Etoposide resulting from the reaction of 2f CH₂Cl₂:MeOH 9:1 (v/v); and for all other phenols resulting from the other compounds 2 CH₂Cl₂:MeOH 9.8:0.2 (v/v). The detection used UV light for 5 and 6 (fluorescence at 360 nm) or the quenching of the fluorescent plate background at 254 nm for phenols. Alternatively for phenols the plates were sprayed with a FeCl₃ solution resulting in colored spots for phenols.

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