Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 1, pp. 41–47, 1995

0968-0896/95 $9.50 + .00

0968-0896(94)00140-5

Molecular Associations of Flavins with Betacarbolines and Related Indoles

Maria A. Muñoz, Carmen Carmona, José Hidalgo, Pilar Guardado and Manuel Balón*
Departamento de Química Física, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

Abstract—The interactions of a set of structurally selected betacarbolines (BC), (9*H*-pyrido[3,4-*b*]indoles) and indoles (IND) with two representative flavins (FN): riboflavin (RFN) and flavin mononucleotide (FMN) have been investigated by absorption and fluorescence spectroscopies. Spectral results provided evidence on the formation of 1:1 non-fluorescent molecular complexes, whose stability constants and other related thermodynamic parameters have been estimated from Stern–Volmer quenching analysis. The FMN complexes are somewhat more stable than the RFN complexes. The stabilities of the IND and BC complexes for a given FN follow approximately the order IND ~ tetrahydro BC < dehydro BC < fully aromatic BC. Protonation of the pyridinic nitrogen atom of BCs has a destabilizing effect, which is more pronounced for fully aromatic than for tetrahydro derivatives. The influence of structural factors on the stability of the complexes has been discussed and, aided by theoretical AM1 calculations, a qualitative model for the structure (stacking) and binding forces (cooperative localized charge transfer and dispersion forces) of the complexes has been proposed.

Introduction

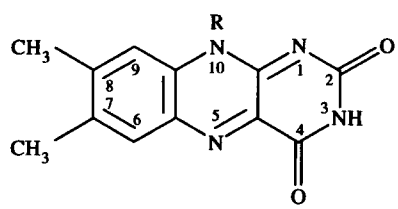
Monoamine oxidase (MAO) is a flavoenzyme that catalyzes the oxidative deamination of important biogenic amines.[1,2] In recent years considerable efforts have been directed to understand the mechanism of both catalysis and inhibition of this enzyme. This interest has been stimulated because MAO inhibitors display important pharmacological properties related to their use as medicinal agents in the treatment of depression[3] and Parkinson's disease.[4] Among the antidepressant drugs, betacarbolines (BC) (9*H*-pyrido[3,4-*b*]indoles) are known to be potent, short-acting and reversible MAO inhibitors.[5]

Kinetics and QSAR studies indicate that BCs compete with the amine substrates for the active site of MAO.[6–8] However, the mechanism at the molecular level of this competitive inhibition still remains unknown. Tomás and Aulló,[9] using a quantum chemical approach, have suggested that BCs and MAO form a reversible complex in which the molecules are linked by two different interactions. The first one arises from a molecular $a\pi - b\pi$ electronic interaction between the BC indole fragment and a suitable region of MAO and, the second one involves the electrostatic interaction of the pyridinic nitrogen atom of BC and an electrophilic center of the enzyme.

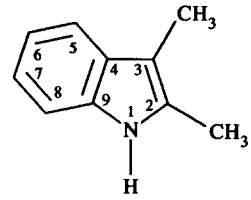
Some authors[10–12] have suggested that the inhibition of MAO by BCs could be related with the known ability of these compounds to form molecular complexes with flavins (FN) (7,8-dimethyl-10-alkylisoalloxazines), the prosthetic group of MAO. Unfortunately, in spite of the potential interest of the FN–BC complexes in relation to the MAO–BC inhibition mechanism, they have been scarcely investigated and, therefore, very little is known about the structure and binding forces of the complexes. Since INDs, the main molecular fragment in BC ring, are also known to form complexes with FNs,[13] it can be supposed that the complexing ability of BCs could be related with the presence of the IND moiety in the BC skeleton. However, there are noticeable differences between the stabilities of the FN complexes of INDs and BCs. Thus, FN–BC complexes are much more stable than FN–IND complexes. Supposedly, the different stabilities could be attributable to an additional and cooperative involvement of the pyridinic nitrogen atom of BCs in the binding forces of the FN–BC complexes, since carbazole, lacking this atom and pyridine, lacking the indole ring, do not form complexes with FNs.[13]

Little work has been reported regarding the influence of structural factors on the stabilities of FN–BC complexes. Furthermore, although aromaticity and protonation of the pyridinic ring in BCs are the main factors determining the reactivity of BCs,[14] the influence of these factors on the FN–BC complexes has not been examined. Nevertheless, a structure–activity study of these structural factors should be a very useful way to investigate the nature of FN–BC complexes. Additionally, the information obtained from this study could clarify the knowledge on the inhibition mechanism of MAO by BCs.

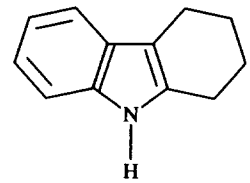
To ascertain the influence of aromaticity and protonation of BC rings on their complexes with FNs, we used steady-state fluorescence quenching experiments to measure the stability constants of the complexes formed by the INDs and BCs shown in Figure 1 with two representative FNs, riboflavin (RFN)



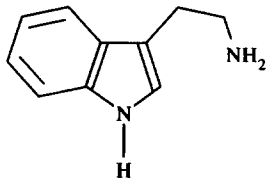
(1) RFN R=-CH_2-$(CHOH)_3$-CH_2OH
(2) FMN R=-CH_2-$(CHOH)_3$-$CH_2OPO_3^{2-}$



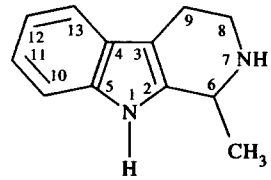
(3) 2, 3-Dimethylindole



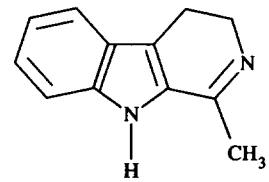
(4) Tetrahydrocarbazole



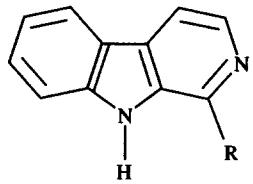
(5) Tryptamine



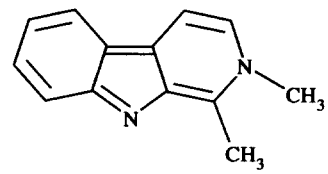
(6) Tetrahydroharmane



(7) Dihydroharmane



(8) Norharmane R= -H
(9) Harmane R= -CH_3



(10) Npy-methylharmane

Figure 1. Structural formulae and numbering system of the FNs, INDs and BCs employed in the present study.

and flavin mononucleotide (FMN). To aid the interpretation of these results, time resolved fluorescence, absorption spectroscopy and AM1 theoretical calculations have also been used as complementary techniques.

Results

As was noted by earlier authors,[10–13] with increasing amounts of INDs or BCs the characteristic absorption band of FNs at ~ 450 nm is progressively shifted and intensified, whilst isosbestic points appear in the spectra. Absorption difference spectra of the mixtures show a broad band centered around 490–500 nm. The broadness of these bands makes the determination of their λ_{max} difficult and, therefore, it precludes comparison among the different complexes.

On the other hand, FN fluorescence at 530 nm (λ_{exc} = 450 nm) is quenched by INDs and BCs. At this wavelength neither the IND nor the BC quenchers show emissions which could interfere with the measurement of FN fluorescence. The quenching of the FN fluorescence occurs without any shift of the emission spectra as shown typically for the **1–9** system in Figure 2. The results obtained for the different FN–IND and FN–BC systems were analyzed according to the classical Stern–Volmer equation:

$$F/F_o = 1 + K_{SV}\,|Q|, \qquad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher at concentration $|Q|$, respectively, and K_{SV} is the quenching constant. Under our experimental conditions, Stern–Volmer plots were always linear for all the systems studied. In every case, the Stern–Volmer constants decreased with increasing temperature and methanol proportion. Some representative Stern–Volmer plots are shown in Figure 3 and K_{SV} constants are summarized in Table 1.

Quenching phenomena can occur either by interaction between the FN and the quencher in the ground state to form a non-excitable complex (static quenching) or by molecular interactions at the singlet excited state of the FN (dynamic quenching).[17] Although the negative temperature coefficients observed in the quenching of FN by INDs and BCs indicate that we are primarily dealing with a static quenching mechanism, it does not exclude the simultaneous occurrence of dynamic quenching process. Therefore, to test this possibility we have measured the fluorescence lifetimes of RFN and FMN in the absence and presence of varying concentrations of representative BC quenchers. The data in Table 2 show the small influence of the quenchers on the fluorescence lifetimes of the FNs. This demonstrates that, at least under our experimental

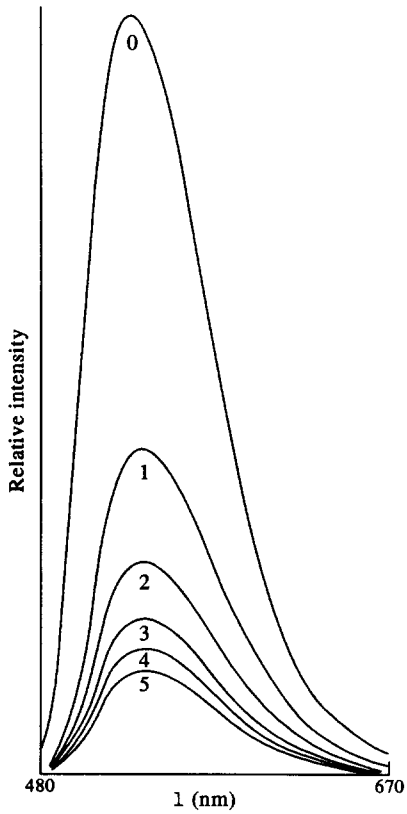


Figure 2. Quenching of the RFN fluorescence by increasing amounts of **9**.

conditions, dynamic processes contribute very little to the total quenching. Similar results were obtained earlier for FN–IND systems.[18]

In addition to fluorescence measurements we have also used absorption spectra to obtain direct information at the ground-state of several representative complexes. In these experiments the absorbances of FN solutions containing varying quencher concentrations ($|FN| << |Q|$) were measured at λ_{max} of their difference spectra. The stability constants of the complexes were evaluated from the following equation that assumes 1:1 stoichiometric binding:

$$\Delta A / [Q] = -K_g \Delta A + K_g \Delta A_c, \quad (2)$$

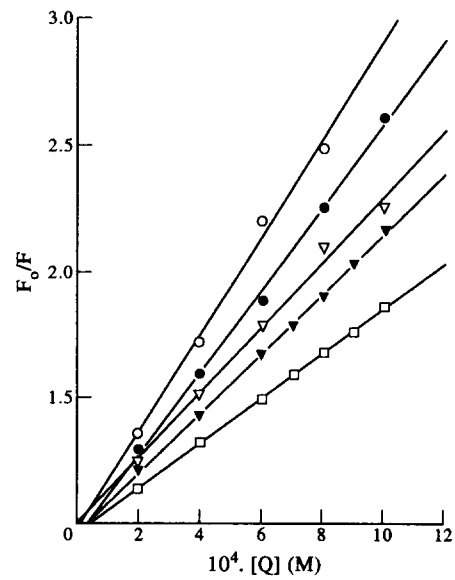


Figure 3. Stern–Volmer plots of the RFN–**9** system at different temperatures.

Table 1. Stern-Volmer quenching constants, Ksv (M^{-1}), of the FN fluorescence by INDs and BCs

			RFN		FMN	
Compound	%MeOH	T(°C)	Cation	Neutral	Cation	Neutral
(3)	10	16.5		185±13		
		20.0		180±12		
		25.0		167±11		190±12
		29.5		153±5		
		36.0		148±23		
	50	25.0		96±7		92±6
(4)	10	25.0		198±19		192±36
(5)	10	25.0	107±5	191±7	146±10	272±9
(6)	10	16.0	164±6			
		20.0		190±9		
		20.5	144±7			
		25.0	133±6	165±12	161±10	268±15
		34.4	111±8			
		35.4		139±3		
		36.0		138.9		
	50	25.0	36±7	78±5		
(7)	10	25.0	168±5	900		
(8)	10	25.0	212±7	660±5	338±11	778±22
(9)	10	15.0		1945±405		
		16.0	480±16			
		20.0		1652±137		1823±58
		20.5	423±15			
		22.5				1651±15
		25.0	372±15	1270±128	483±15	1410±38
		30.0		1159±27		1136±18
		34.4	288±19			926±34
		35.0		897±23		926±34
	50	25.0	111±11	58±4		
(10)	10	25.0	226±7	968±15	308±9	715±20

Table 2. Fluroescence lifetimes of FNs in the presence of varying concentrations of representative quenchers

	τ /ns
RFN (4×10^{-5} M, 10% MeOH, pH=5)	4.5
+ (9) (4×10^{-5} M)	4.5
+ (9) (1.2×10^{-4} M)	4.4
+ (9) (8×10^{-4} M)	4.4
+ (6) (1.2×10^{-4} M)	4.5
RFN (4×10^{-5} M, 10% MeOH, pH=9)	4.4
+ (9) (1.2×10^{-4} M)	4.3
+ (9) (8×10^{-4} M)	4.3
+ (6) (1.2×10^{-4} M)	4.5
FMN (4×10^{-5} M, 10% MeOH, pH=9)	4.7
+ (9) (1.2×10^{-4} M)	4.6
+ (9) (8×10^{-4} M)	4.4
+ (6) (1.2×10^{-4} M)	4.6

where ΔA is the apparent absorption change at the titration wavelength relative to the completely free FN, and ΔA_C is the corresponding absorption change for the complexed FN. Illustrative results of these calculations are reported in Figure 4 and Table 3. From these results two conclusions emerge; firstly, the linearities of these plots indicate a 1:1 stoichiometry for the complexes. Secondly, the close correspondence between the stability constants determined by absorption and fluorescence methods corroborates the quenching of the FN fluorescence by BCs is primarily due to the ground-state formation of 1:1 non-fluorescent complexes. Furthermore, under our experimental conditions, the collisional quenching contribution to K_{SV} is masked by the experimental errors inherent to the determination of stability constants. On this assumption, we will use K_{SV} constants in Table 1 as a direct measure of the stability constants of the complexes.

The data in Table 1 document the influence of different structural factors among INDs and BCs on the stability constants of their FN complexes. Thus, it can be noted that, for a given IND or BC, the FMN complexes are usually more stable than the RFN ones and, for a given FN, the stabilities of the neutral complexes roughly follow the order: INDs ~ tetrahydro BC < dihydro BC < fully aromatic BCs. On the other hand, protonation of the non-indolic nitrogen atom of **5** and BCs, has a destabilizing effect. This effect is much more pronounced for the fully aromatic BCs **8** and **9** than for the less aromatic member **6**. Table 1 also contains information about the influence of methanol on the stability constants of some selected complexes. It can be observed that the increase of methanol proportion produces a decrease in the stability of the complexes. Again, this effect is more pronounced for the fully aromatic than for the less aromatic members of the BC series.

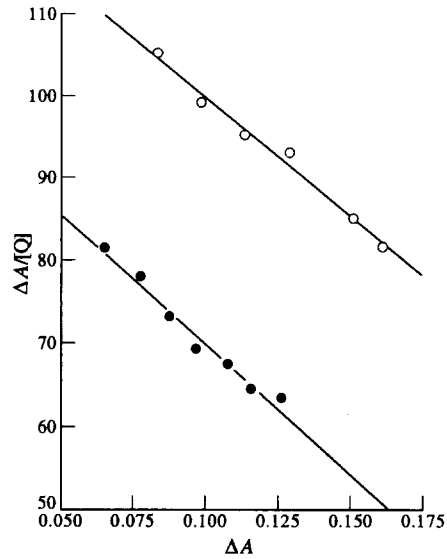


Figure 4. Benesi–Hildebrand plots of several representative systems.

Table 3. Ground state formation constants, K_g (M^{-1}), of several representative complexes determined by absorption spectroscopy at 25 °C

System	pH	K_g (M^{-1})
RFN–(5)	9	154±20
RFN–(6)	9	148±15
RFN–(9)	5	382±26
RFN–(9)	9	1382±110
FMN–(9)	5	408±45
FMN–(10)	9	779±121
FMN–(10)	5	306±59

Since van't Hoff plots of ln K_{SV} vs $1/T$ gave good straight lines, they were used to obtain information on the thermodynamic parameters for the formation of several representative complexes. These data show that enthalpy is the principal factor in determining the stabilities of the complexes. This would suggest that hydrophobic interactions are not playing an important role in the binding forces of the complexes. The positive ΔS associated to the formation of the RFN–**3** complex may be a result of loss of solvation in the formation of this complex. Solvation would decrease as the ring size increases from INDs to BCs and, therefore, this effect would be cancelled by the loss of conformational entropy involved in the formation of BC–FN complexes.

Finally, in order to assess the possibility of electrostatic interactions in the complexes, additional experiments varying the ionic strength by the addition of NaCl and $MgCl_2$ were carried out. Ionic strength does not appreciably affect the stability of the RFN–**9** complex (Table 5), but it has a clear destabilizing effect on the FMN–**9** complex. This effect is greater for cationic than for neutral species and it is also more pronounced for $MgCl_2$ than for NaCl. The greater salt effect of Mg^{2+} relative to Na^+ seems to be not only a result of its

Table 4. Thermodynamic parameters associated with the formation of some representative complexes of FNs with INDs and BCs

	RFN–(3)	RFN–(6)		RFN–(9)		FMN–(9)
	Neutral	Cation	Neutral	Cation	Neutral	Cation
ΔG /kJ.mol^{-1}	−12.7±0.2	−12.1±0.1	−12.7±0.2	−14.7±0.1	−18.0±0.4	−18.0±0.1
ΔH /kJ.mol^{-1}	−9±1	−15±3	−15±2	−20.6±0.2	−29±5	−34±7
ΔS /J.K^{-1}mol^{-1}	+12±2	−11±5	−8±4	−19.8±0.5	−37±9	−54±12

greater influence on the ionic strength, but also to the known ability of magnesium cations to specifically interact with phosphate anions by forming ion-pairs. We attribute the above results to the existence of electrostatic interactions between the phosphate group of FMN and the positively charged pyridinic nitrogen atoms of Bcs.

Discussion

The results of the present study conclusively demonstrate that INDs and, independently of their degree of aromaticity and protonation state, BCs interact with FNs to form 1:1 non-fluorescent molecular complexes. Owing to the related structures of the substrates and to the similar spectral characteristics of the complexes, it seems reasonable to consider that some common binding forces operate in the INDs and BCs complexes with FNs. The different stabilities among the FN complexes of INDs and BCs could be due to the different intensities of the common forces, or, alternatively, to the existence of other cooperative forces which do not operate in the FN–IND complexes.

Table 5. Influence of the addition of NaCl and $MgCl_2$ on the formation constants (M-1) of RFN and FMN complexes of **9** in 10% v/v methanol-water at 25 °C

Added Salt (M)	RFN		FMN	
	Cation	Neutral	Cation	Neutral
NaCl				
0.00	372±5	1270±28	483±15	1410±38
0.25	351±11	1269±20	390±8	1393±23
0.50	361±7	1255±14	351±9	1355±14
$MgCl_2$				
0.025			412±8	
0.10			341±22	
0.20	345±12	1240±17	323±20	1315±18

With respect to the latter possibility, the hypothesis of a direct involvement of the BC pyridinic nitrogen atom in the binding forces of the FN–BC complexes is very suggestive. Thus, the lone electron pair on this atom in the neutral BCs, provides to these molecules a potential n-donor site which is absent in IND derivatives. In fact, protonation of this atom produces a pronounced destabilizing effect in the complexes. However, the complex of the neutral N_{py}-methyl derivative **10** with RFN is less stable than that of **9** but more stable than that of **8**. This sequence is not that expected if their pyridinic nitrogen atoms were directly involved in the binding forces of these complexes. On the other hand, compounds such as **5** and **6**, which possess nitrogen atoms with easily available electron pairs, would form flavin complexes more stable than those of the typical INDs **3** and **4**. All the above considerations lead us to conclude that the pyridinic nitrogen atoms of BCs are not directly involved in the binding mechanism of FN–BC complexes.

The observed increase in the stability of the BC complexes with increasing aromaticity of the BC ring and the decrease in entropy observed on complexing indicate a stacked structure for the complexes. On the other hand, the destabilizing effect of methanol is also in accord with earlier results on related stacking interactions, namely the association invariably decreases with addition of organic co-solvent to water.[13,19] Since stacking interactions are significantly dependent upon the aπ–bπ orbital interaction, an investigation of the HOMOs of INDs and BCs might lead to a better comprehension of the nature of their FN complexes. These calculations were carried out by the semi-empirical AM1 method and the results for representative structures are given in Table 6. Previously, we have successfully used the AM1 method to describe the geometries, energies and reactivities of a set of structurally related BCs.[14,20,21]

Data in Tables 1 and 6 show the existence of two roughly linear correlations between the HOMO energies of the charged and neutral BC molecules and the stability constants of their FN complexes. INDs do not match as well as BCs in these correlations. However, it is well known that this type of analysis is very sensitive to the atomic basis set and, therefore, a direct comparison between the HOMO energies of INDs and BCs cannot be made.

The above relationships indicate that the predominant factor in the stabilization of the complexes is a donor–acceptor interaction involving the π-electron systems of the IND or BC substrate and the FN molecule. Therefore, we can assume two modes of binding as the main possible contributors to the overall stability of the stacked complexes: charge transfer (CT) and π-electron dispersive interactions. Furthermore, electrostatic interactions, as we have previously suggested, also play a role in the stability of the FMN complexes.

Table 6. Theoretical AM1 calculations on INDs and BCs

	Cation			Neutral		
Compound	HOMO[a]	f_r[b]	D_e[c]	HOMO	f_r	D_e
(3)				-8.134	C-3: 0.52	C-3: 4.14
(4)				-8.087	C-3: 0.52	C-3: 4.14
(5)	-11.195	C-3: 0.50	C-3: 4.22	-8.288	C-3: 0.51	C-3: 4.14
(6)	-11.558	C-10: 0.42	C-3: 4.13	-8.122	C-3: 0.54	C-3: 4.14
(7)	-12.148	N-1: 0.37		-8.296	C-3: 0.49	N-1: 5.19
						C-3: 4.11
(8)				-8.535	N-1: 0.50	N-1: 5.23
(9)	-12.269	N-1: 0.39	N-1: 5.20	-8.429	N-1: 0.48	N-1: 5.23

a) HOMO energies in eV.
b) Frontier electron densities: $f_r = [2 \times (C^{HOMO})^2]$.
c) Absolute electronic densities.

The CT nature of the IND–FN complexes has been the subject of much controversy. Thus, as it is expected for CT complexes, their stabilities strongly depend on the presence of electron donating or electron withdrawing groups on the indole ring[18] and they also exhibit a new absorption band which is intensified upon decreasing temperature. However, conversely to that expected for typical CT complexes, the correlation between the ionization potentials of the donors and the λ_{max} of these complexes is not observed.[13,17] Thus, as it has been suggested[22–24] the strong CT donor ability of INDs arises from the presence in this ring of strong negatively charged atoms rather than from the π-donation of the IND conjugated system. This hypothesis is supported by crystallographic studies on the FN–tryptophan complexes.[25]

Owing to the close similarity among the IND and BC complexes, it is reasonable to suggest that localized CT interactions could also operate in the binding mechanism of BC–FN complexes. These interactions would occur when the atom in the BC ring with the higher electronic density is aligned with the atom with the largest positive charge density of the isoalloxazine ring, i.e. the N-1 atom of the FN molecules.[26] It can be supposed that the orientation of the molecules in the complex would be governed to a considerable degree by the matching of the charge complimentarity necessary for the CT interaction, so as to maximize the π-electronic dispersive interactions.

Interestingly, frontier electron indices and absolute electronic densities of single atoms in BC rings (Table 6) suggest that depending on the aromaticity degree of the BC substrate, the most favorable sites for CT interaction and, therefore, the geometry of the complexes would be different. Thus, in the case of the tetrahydro BC system, the HOMO is a π-orbital quite similar to that of the indole molecule and as in these compounds the electrophilic attack is directed towards C-3. In contrast, the fully aromatic BC has a π-HOMO where the charge is redistributed along most of the atoms of the three rings thus favoring the N-1 position. The behavior of the dihydro compound is more closely related to that of the fully aromatic than to the tetrahydro derivative, being the N-1 position also favored for this compound.

The different arrangements of the BC and IND rings in the complexes implicate different overlapping with the isoalloxazine ring of the FNs and, therefore, different magnitude for the dispersive forces. Intuitively one would expect that, owing to their extended π-electron system, fully aromatic BCs overlap more extensively with the isoalloxazine ring than the IND derivatives do. Molecular electrostatic potential (MEP) maps support this suggestion.[12,26,27]

The idea of different structures for the FN–BC complexes depending on the aromaticity of the BC ring has been previously suggested to account for the different influence that methyl substitution exerts on the inhibitory potency of tetrahydro and fully aromatic BCs.[7] Finally, although at this point we think that any parallels drawn between FN–BC complexes and MAO–BC inhibition must be considered speculatively, we want to point out that the sequence of the stability constants of the FN–BC complexes closely follows the inhibitory activity towards MAO. On the other hand, the supposed structure of the complexes could explain many characteristics of the competitive MAO–BC inhibition mechanism. Thus, the current working hypothesis for enzymatic MAO mechanism,[28] involves an initial electron transfer step in which the amine substrate serves as the electron donor and the MAO flavin residue plays the role of an electron acceptor. BCs can compete with the amine by the MAO flavin residue and mimic the electron transfer process blocking the active center of the enzyme.

Experimental

Compounds **1–5**, **8** and **9** were commercial products (Sigma, Aldrich) of the best available quality (> 98%) and were used as received. BCs **6**, **7** and **10** were synthesized as in previous works.[15,16]

Absorption spectra were run on a Hewlett Packard 8452A Diode Array spectrophotometer. Steady-state fluorescence measurements were carried out on a Perkin Elmer 650-40 spectrofluorometer. Fluorescence lifetimes were measured on an EEY nanosecond fluorometer equipped with a hydrogen lamp (10 atm) and operating in the time correlated single photon counting mode.

Solutions for spectroscopic measurements were prepared by suitable dilution of a daily prepared solution of the FN with 20% v/v methanol–water buffered solutions of the IND or BC substrate. Acetic acid–sodium acetate (pH ~ 5) and boric acid–sodium tetraborate (pH 8–9) were used as buffers to ensure the exclusive formation of neutral and cationic BC species. Pyridinic protonation pK_as of BCs ranged between 6 and 8.[15,16] Final solutions contained fixed concentration of the FN ($\sim 4 \times 10^{-5}$ M), varying concentrations of the IND or BC substrate (10^{-4}–10^{-3} M) and 10% v/v of methanol. The ionic strength of the solutions was 0.050 M.

Semiempirical calculations at the AM1 level were carried out as in previous work.[14]

Acknowledgements

The financial assistance of the Dirección General de Investigación Científica y Técnica (PB92-0686) and Junta de Andalucia is appreciated. The authors are also very much indebted to Professor Bartolomé Quintero for his assistance in performing the fluorescence lifetime measurements.

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(*Received in U.S.A.* 24 *June* 1994; *accepted* 22 *September* 1994)