Substituent and Steric Effects of Flavin Models in the Reactions of *N*-Benzyl-1,4-dihydronicotinamide, Butane-1,4-dithiol, Phenylhydrazine, and Nitroethane

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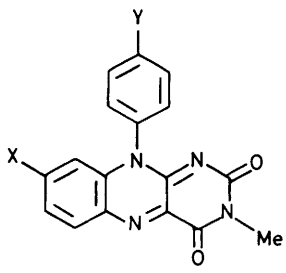
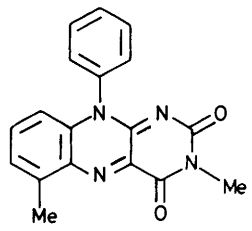
Reactivities of 3-methyl-10-phenyl-8-substituted-, 3-methyl-10-(*p*-substituted phenyl)-, and 3,6-dimethyl-10-phenylisoalloxazines have been kinetically investigated for oxidations of *N*-benzyl-1,4-dihydronicotinamide, $HS(CH_2)_4SH$, $PhNHNH_2$, and $EtNO_2$ in EtOH containing diazabicycloundecene as a base or in aqueous solution under anaerobic conditions. Substituent effects and the steric hindrance of the 6-methyl group are discussed in connection with the reaction mechanisms.

Biomimetic investigations of flavin coenzymes have received considerable attention including physical organic chemical studies.[1] Nevertheless, it is not easy to determine the mechanisms of flavin-mediated reactions, probably because the isoalloxazine nucleus has multiple reaction sites, and undergoes both one- and two-electron transfer reactions. Investigations of structurally modified flavin analogues such as 5-deaza-,[2] 10-thia-,[3] and 5-alkyl-flavins[4] have given much information about the parent flavin. To our surprise, however, classical approaches such as measurement of substituent and steric effects of the isoalloxazine ring seem to be lacking, although electron-deficient flavins such as the 8-cyano-[5] and the 8-aza-flavins[6] have been known to enhance the oxidizing activity. Bruice *et al.* have employed flavins bearing 10-(2,6-dimethylphenyl) and 6,8-sulphonic acid moieties for sulphite addition[7] and for hydrolysis[8] of the isoalloxazine nucleus for the investigation of the mechanisms. In the former flavin, the 9a and 10a positions are considered to be shielded by the 2'- and 6'-methyl groups, and in the latter, the N(5) position is sterically crowded due to the 6-sulphonate system. These scattered data allow us to say that substituent and steric effects of the isoalloxazine ring are quite useful for the determination of the mechanisms of flavin-mediated reactions.

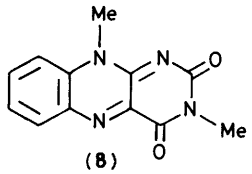
In this paper, we describe the kinetic studies of the title compounds by employing substituted isoalloxazines in EtOH containing diazabicycloundecene (DBU) except for *N*-benzyl-1,4-dihydronicotinamide (BNAH) oxidation (in aqueous solution) under anaerobic conditions, and discuss the relation between the reaction mechanisms and substituent and steric effects.



(1) X = H, Y = H
(2) X = Me, Y = H
(3) X = OMe, Y = H
(4) X = Cl, Y = H
(5) X = H, Y = OMe
(6) X = H, Y = Cl



(7)



(8)

Results and Discussion

Flavins (1)—(6) were synthesized and employed for the reactions.

Oxidation of BNAH.—Oxidation of *N*-substituted 1,4-dihydronicotinamide by flavins has been extensively investigated, since it is an important reaction in the respiratory chain. The oxidation mechanism seems to proceed *via* hydride ion transfer passing through pre-equilibrium complexing of the dihydropyridine and isoalloxazine rings.[9]

The rate constants were determined spectrophotometrically by following the absorption decreases of the oxidized flavins (440 nm) in aqueous solution under anaerobic conditions. The rates followed first-order kinetics up to more than 90% of reaction. The formation of 1,5-dihydroflavins was confirmed by quantitative regeneration of the oxidized flavins by O_2 introduction. The results are shown in Table 1.

Although the number of the substituents is few, the Hammett ρ values were determined by the method of least squares to be ρ_8 2.0 (γ 0.96) for the 8-position, and ρ_{10} 0.4 (γ 0.98) for the 10-position, respectively. The reaction site of the isoalloxazine ring is the N(5) atom which is *para* to the 8-position. Thus, the ρ_8 value (+2.0) suggests that a hydride ion from BNAH is partially transferred to the N(5) position of the isoalloxazine ring in the transition state, as proposed in the reaction of isoquinolinium ion with 1,4-dihydronicotinamide derivatives.[10] The smaller ρ_{10} value appears to support this mechanism. The rate of (7) could not be determined due to its unexpected low solubility in water.

Oxidation of Butane-1,4-dithiol.—We have chosen the oxidation of thiols by flavins, since the mechanism is well established in model systems. The reaction involves nucleophilic attack of a thiol anion at the C(4a) position to form a covalent adduct followed by nucleophilic attack of the second thiol anion to afford the corresponding disulphide and 1,5-dihydroflavin in aqueous solution.[11] The rate-determining step is the nucleophilic attack at the C(4a) position for the oxidation of dithiols, and the subsequent thiol attack for the oxidation of monothiols. Thus, this is a good reaction for estimating the substituent and steric effects for reactions proceeding *via* 4a-adduct formation. However, the rates of the oxidation by conventional flavins are known to be very slow in aqueous solution.[5,6] Thus, we have employed EtOH containing DBU as the reaction system. It was confirmed that the reaction of

Table 1. Rate constants for BNAH oxidation[a]

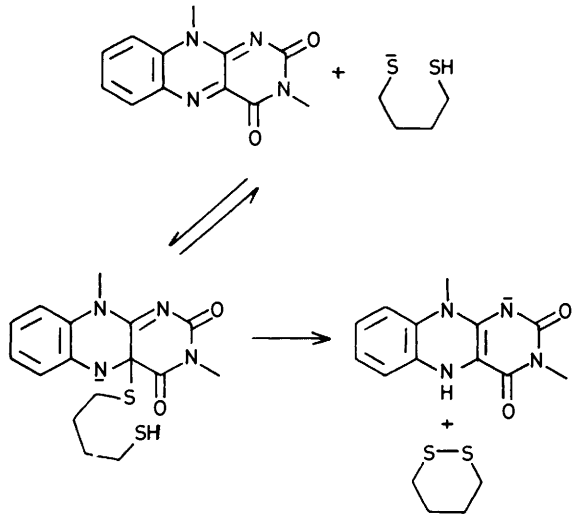
Flavin	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
$10k_{obs}/min^{-1}$	5.18	2.27	0.852	9.32	3.85	5.87	*b*	8.41

[a] [Flavin] 5×10^{-5}M, [BNAH] 1×10^{-3}M, pH 9.27 (0.1M borate, μ 0.5), 25 °C. [b] The rate could not be determined due to solubility problems.

Table 2. Rate constants for $HS(CH_2)_4SH$ oxidation[a]

Flavin	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
k_{obs}/min^{-1}	4.65	0.575	0.0194	13.0	3.30	7.28	0.627	0.934

[a] [Flavin] 5×10^{-5}M, [$HS(CH_2)_4SH$] 3.0×10^{-3}M, [DBU] 1.5×10^{-3}M in EtOH at 25 °C.



Scheme 1.

$HS(CH_2)_4SH$ with (**8**) gives tetramethylene disulphide and the 1,5-dihydroflavin in EtOH–DBU.

The rate constants were determined spectrophotometrically by following the absorption decrease of the flavins under anaerobic conditions. All the reactions followed first-order kinetics up to more than two half-lives. The oxidized flavins were quantitatively recovered by the introduction of O_2 for all cases. This indicates that thiol oxidation by the 8-chloroflavin (**4**) is much faster than the substitution of the 8-chloro group by the thiol anion. However, a characteristic absorption spectrum (490 nm) of an 8-alkylthioflavin was observed after a few hours in an open cuvette. A facile nucleophilic substitution of the 8-chloro group of the isoalloxazine ring is a well known phenomenon.[12] The rate constants of the oxidation are presented in Table 2.

The ρ values were determined: ρ_8 5.2 (γ 0.99) and ρ_{10} 1.0 (γ 0.99), respectively. These values are much larger than those for the BNAH oxidation. The large ρ_8 value may be best accounted for by the fact that almost full negative charge, which is produced by nucleophilic attack of the thiol anion at the C(4a) position, develops at the N(5) atom in the transition state, suggesting 4a-adduct formation to be rate determining (Scheme 1). No rate retardation due to the 6-methyl group was observed [(**2**) *versus* (**7**)]. This steric hindrance was not observed also for the oxidation of thiophenol (Table 3).

The foregoing results imply that reactions proceeding through 4a-adduct formation may display a relatively large ρ_8 value and may suffer no steric hindrance of the 6-methyl group of the isoalloxazine ring. Thus, it is of interest to establish whether this concept is generalized in flavin model reactions. Thus, we have examined other oxidation reactions which may proceed *via* covalent adduct formation, although the mechanisms have not been established in model systems.

Table 3. Rate constants for PhSH oxidation by (**2**) and (**7**)[a]

Flavin	(2)[b]	(7)[b]
$10k_{obs}/min^{-1}$	1.76 ± 0.05	2.52 ± 0.03

[a] [(**2**)] = [(**7**)] = 5×10^{-5}M, [PhSH] 2×10^{-2}M, [DBU] 1×10^{-2} in EtOH at 25 °C. [b] Average value of two runs.

Oxidation of Phenylhydrazine.—The reaction of hydrazine derivatives with flavins is biochemically important, since they are known to be suicide inhibitors for mammalian monoamine oxidase[13] and bacterial trimethylamine dehydrogenase.[14]

The reaction of $PhNHNH_2$ with flavins was also performed in EtOH containing DBU, since the reaction did not occur in aqueous solution. The spectroscopic examination of the reaction of $PhNHNH_2$ with (**8**) in EtOH–DBU showed the formation of the 1,5-dihydro-form of (**8**) which regenerated the oxidized form quantitatively by introduction of O_2. The formation of phenyldiazene (PhN=NH) was confirmed by detecting benzene as its decomposition product, since phenyldiazene is known to be unstable under these conditions.[15]

The rate constants were determined as for the thiol oxidation (Table 4). The rate of 8-chloroflavin (**4**) could not be determined due to the substitution of the chloro group by $PhNHNH_2$, which was confirmed by the presence of the characteristic absorption spectrum of an 8-alkylaminoflavin.[1a] Thus, the Hammett ρ values were determined to be ρ_8 4.9 (γ 0.98) from (**1**)—(**3**), and ρ_{10} 0.9 (γ 0.90), respectively. Table 4 shows that the 6-methylflavin does not reduce the rate [(**2**) *versus* (**7**)]. These results are quite similar to those for the thiol oxidation. The similar ρ values and no rate retardation due to the 6-methyl group strongly suggest that both reactions proceed *via* similar mechanisms. To confirm this, further kinetic studies were carried out. The rates were found to be first order in the concentrations of $PhNHNH_2$ and DBU, respectively (Figures 1 and 2). It was also found that $PhNHNH_2$ reacts 60 times faster with (**8**) than does $MeNHNH_2$, and (**8**) does not react at all with Me_2NNH_2 under the conditions of Table 4. Thus, it can be said that the α-hydrogen atom of hydrazines plays a crucial role in hydrazine oxidation. These observations may be best accounted for by the mechanism in Scheme 2. $PhNHNH_2$ attacks the C(4a) position of the isoalloxazine ring to form the 4a-adduct followed by β-elimination to afford the 1,5-dihydroflavin and PhN=NH. The observed rate constant ($k_{obs.}$) is expressed by equation (1), by assuming a steady state for the concentration of

Table 4. Rate constants for $PhNHNH_2$ oxidation[a]

Flavin	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
$10k_{obs}/min^{-1}$	2.09	0.501	0.0091	*b*	1.84	5.10	0.456	2.39

[a] [Flavin] 5×10^{-5}M, $[PhNHNH_2]$ 2×10^{-3}M, [DBU] 5×10^{-3}M in EtOH at 25 °C. [b] The rate could not be determined due to substitution of the 8-chloro group.

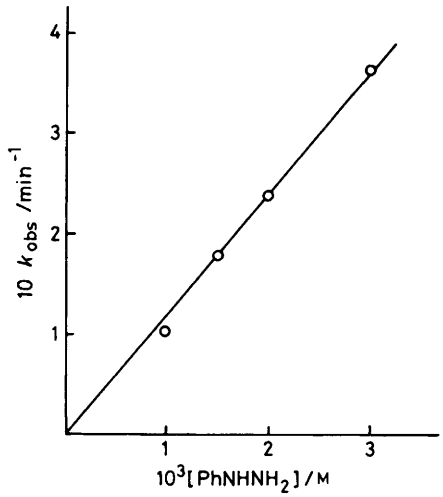


Figure 1. Effect of concentration of $PhNHNH_2$: [(**8**)] 5×10^{-5}M, [DBU] 5×10^{-3}M, EtOH, 25 °C

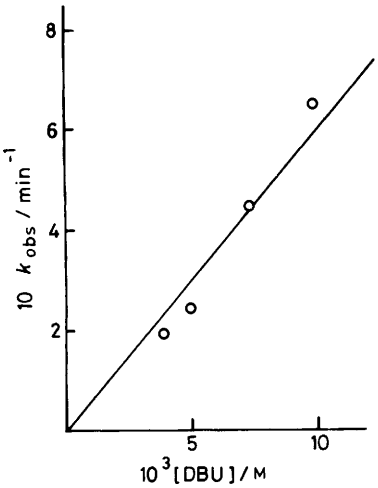
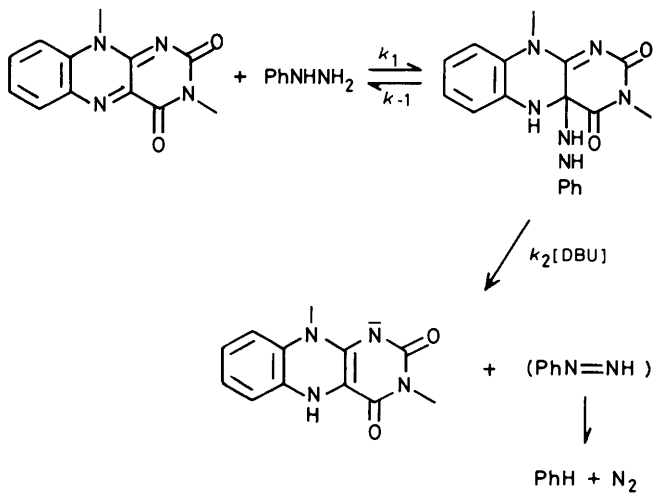


Figure 2. Effect of concentration of DBU: [(**8**)] 5×10^{-5}M, $[PhNHNH_2]$ 2×10^{-3}M, EtOH, 25 °C

the 4a-adduct, where $[Flavin]_o$, $[PhNHNH_2]_o$, and $[DBU]_o$ are initial concentrations. The first-order dependence of the rates

$$k_{obs}[Flavin]_o = \frac{k_1k_2[PhNHNH_2]_o[DBU]_o[Flavin]_o}{k_{-1} + k_1[PhNHNH_2]_o + k_2[DBU]_o} \quad (1)$$

on $[PhNHNH_2]_o$ and $[DBU]_o$ allows us to assume $k_{-1} \gg k_1[PhNHNH_2]_o + k_2[DBU]_o$ to give equation (2). All the data



Scheme 2.

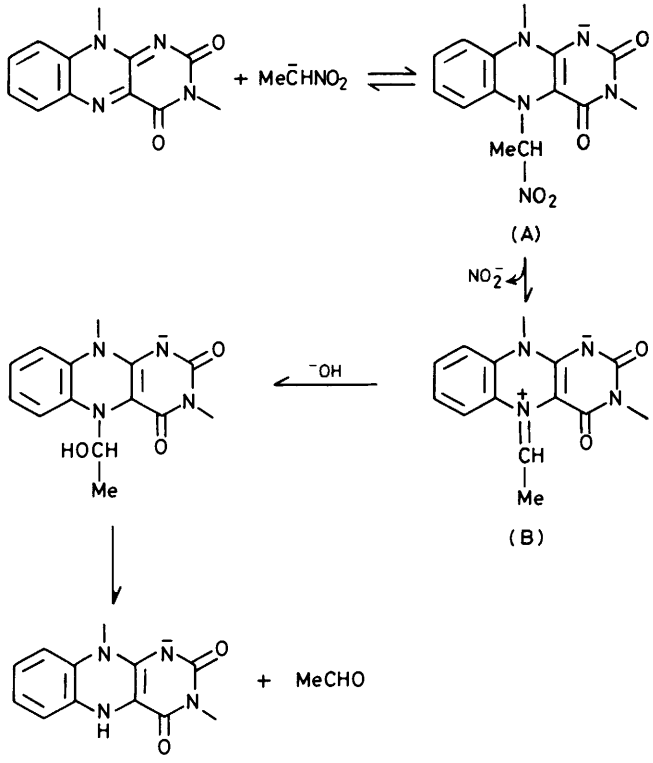
$$k_{obs.} = k_1k_2[PhNHNH_2]_o[DBU]_o/k_{-1} \quad (2)$$

obtained strongly suggest that the oxidation of $PhNHNH_2$ proceeds *via* 4a-adduct formation.

Reaction of Nitroethane.—D-Amino acid oxidase oxidizes nitroethane anion to give acetaldehyde and nitrite ion according to Scheme 3.[16] An important point in Scheme 3 is formation of an N(5)-adduct (A).

In non-enzymatic systems, however, the mechanism of nitroalkane oxidation has not been established. However, oxidation through the 4a-adduct is considered to be highly unlike, since the 4a-adduct formed by nitromethane with 5-ethyl-3-methyl-lumiflavin is known not to give an oxidation product.[4c]

We have again employed EtOH–DBU as the reaction system, since conventional flavins do not react with nitroalkane anion in aqueous solution.[5] Thus, prior to the rate measurement, the reaction of (**8**) with nitroethane in EtOH–DBU was examined spectrophotometrically under anaerobic conditions. If the reaction proceeds according to Scheme 3 in EtOH, the product would be 5-(1-ethoxyethyl)-1,5-dihydroflavin (c) formed by EtO^- attack to the iminium ion (B). The time course of the spectral changes is shown in Figure 3. Figure 3 indicates that the absorption of (**8**) decreases to give a spectrum with a shoulder at *ca.* 350 nm and an isosbestic point at 315 nm. Spectroscopic examination of the product showed: (a) introduction of O_2 did not change the spectrum, (b) acidification by aqueous HCl (1M) regenerated the oxidized flavin, and (c) addition of $NaB(CN)H_3$ followed by introduction of O_2 did not change the spectrum. These observations imply that the product is not (i) 1,5-dihydroflavin based on (a), (ii) 5-(1-ethoxyethyl)-1,5-dihydroflavin (c) based on (a) and (b), and (iii) the iminium ion (B) based on (b) and (c), since the 5-adduct is usually stable to acid[17] and gives a stable flavin radical on admittance of O_2,[4a] and the



Scheme 3.

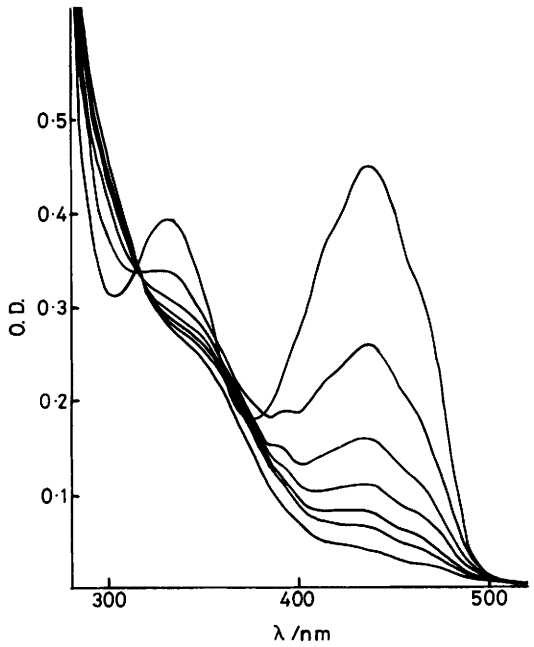
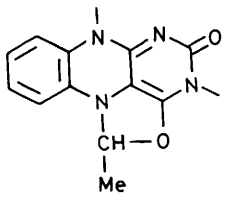


Figure 3. Time course of the spectral changes of the reaction of nitroethane with (**8**): [(**8**)] 5×10^{-5}M, [$EtNO_2$] 1×10^{-2}M, [DBU] 3×10^{-2}M, EtOH, 25 °C

iminium ion (B) is reduced by $NaB(CN)H_3$ to give 1,5-dihydroflavin which yields the stable flavin radical on admittance of O_2.[18] The 4a-adduct is also excluded by (a) and (b).[17] Thus, this reaction helps to establish the mechanism by examining the substituent and steric effects of the isoalloxazine ring.



(D)

The rate of the decrease of the oxidized flavins was found to follow first-order kinetics up to more than 90% of reaction. The rate constants are given in Table 5.

The substituent effects are ρ_8 3.1 (γ 0.99) and ρ_{10} 1.1 (γ 0.90). A relatively large ρ_8 value suggests the reaction site to be C(4a) or N(5) of the isoalloxazine ring. However, considerable rate retardation due to the 6-methyl group [(**7**)/(**2**) = 1/24] strongly suggests the N(5)-position to be the reaction site. The kinetic results together with the spectral behaviour may not be inconsistent with the formation of (D), formed by intramolecular nucleophilic attack of C=O(4) to (A) or (B).

Conclusions.—The substituent and steric effects of the isoalloxazine ring have been examined for the first time. A relatively large ρ_8 value suggests the reaction site to be C(4a) or N(5) and the steric effect of the 6-methyl group plays a crucial role. Examination of both effects could be a convenient method to estimate the mechanism of flavin-mediated reactions.

Experimental

Materials.—Flavins were synthesized according to the method of Yoneda *et al.*[19] Compounds (**1**), (**2**), (**4**), and (**6**) were prepared from 3-methyl-6-arylaminouracils and the corresponding nitrosobenzenes. Identification was performed by elemental analyses (Table 6). Compound (**3**) was prepared by condensation of *N*-methylalloxane and 2-amino-5-methoxydiphenylamine which was prepared from acid-catalysed rearrangement of 4-methoxyhydrazobenzene.[20] Compounds (**7**), (**8**), and BNAH were samples from our previous study.[21] Butane-1,4-dithiol, thiophenol, and nitroethane were purified by distillation under N_2. Phenylhydrazine, methylhydrazine, 1,1-dimethylhydrazine, and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Wako Chemical Co., and used without further purification. Ethanol was purified as described previously.[21]

Kinetics.—The kinetics of BNAH oxidation by flavins was performed as described previously.[22] The concentrations of stock solutions are; flavins (5×10^{-3}M in dimethylformamide), $PhNHNH_2$ (0.2M in EtOH), $HS(CH_2)_4SH$ (0.3M in EtOH), $EtNO_2$ (1.0M in EtOH), and DBU (0.15, 0.5, and 3M in EtOH). A typical kinetic run is as follows. In a Thunberg cuvette, flavin solution (30 μl) was placed in the cell with EtOH (2.91 ml), and substrate (30 μl) and DBU (30 μl) were placed in the upper counterpart. Both the solutions were degassed by bubbling vanadous ion-scrubbed N_2 prehumidifed with EtOH for 20 min. After equilibrium at 25 °C, the reaction was initiated by mixing.

Product Analysis of $PhNHNH_2$ *Oxidation.*—A mixture of (**8**) (20 mM), $PhNHNH_2$ (20 mM), and DBU (20 mM) in diethyl ether (5 ml) was stirred for 3 days in the dark under N_2 at room temperature. The ether was washed with H_2O (5 ml), and dried over Na_2SO_4. The ether layer was subjected to g.l.c. [internal

Table 5. Rate constants for the reaction of $EtNO_2$[a]

Flavin	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
$10k_{obs}/min^{-1}$	5.08	2.42	0.683	29.5	4.33	16.4	0.0998	0.595

[a] [Flavin] 5×10^{-5}M, [$EtNO_2$] 1×10^{-2}M, [DBU] 3×10^{-2}M in EtOH at 25 °C.

Table 6. Analytical data for flavins[a]

Flavin (Formula)	Found (%) (Required) C	H	N
(1)	67.3	3.9	18.7
($C_{17}H_{12}N_4O_2$)	(67.1)	(4.0)	(18.4)
(2)	67.8	4.6	18.0
($C_{18}H_{14}N_4O_2$)	(67.9)	(4.3)	(17.6)
(3)	64.5	4.2	16.45
($C_{18}H_{14}N_4O_3$)	(64.7)	(4.2)	(16.8)
(4)	60.2	3.0	16.5
($C_{17}H_{11}ClN_4O_2$)	(60.3)	(3.3)	(16.5)
(5)	64.5	4.0	16.5
($C_{18}H_{14}N_4O_3$)	(64.7)	(4.2)	(16.8)
(6)	60.2	3.0	16.45
($C_{17}H_{11}ClN_4O_2$)	(60.3)	(3.3)	(16.5)

[a] All flavins were recrystallized from EtOH, and m.p.s were > 330 °C.

standard toluene, column (SF 96, 1 m), oven temperature 50 °C]. Benzene was produced in 15—30% yield.

Acknowledgements

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