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Reaction of amines with N^1,N^{10}-ethylene-bridged flavinium salts: the first NMR spectroscopic evidence of C^{10a} tetrahedral amine adducts

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Abstract—Two different ^{13}C-labeled 7-trifluoromethyl-N^1,N^{10}-ethyleneisoalloxazinium chlorides were utilized to examine the mechanism of amine dehydrogenation. ^1H NMR studies in CD_3CN (^{13}C NMR in DMSO-d_6), as confirmed using ^{15}N-labeled benzylamine, indicate that primary and secondary amines add to give tetrahedral C^{10a} adducts that persist for hours at 25°C. Upon heating, the C^{10a} amine adducts partition between rearrangement to C^{4a} spirohydantoin amidines, and, in the case of benzylic amines, β-elimination to give reduced flavin and imine dehydrogenation product. A C^{10a} tetrahedral hydroxy adduct, generated under basic conditions when water was present, was also confirmed by $^1H/^{13}C$ NMR.

1. Introduction

Monoamine oxidase (EC 1.4.3.4, MAO),[1–5] D-amino acid oxidase (EC 1.4.3.3),[6–8] and sarcosine oxidase[9] carry out the flavin-dependent dehydrogenation of amines and amino acids, yet the mechanisms utilized for dehydrogenation are still unclear. In each case, the tightly- or covalently-bound flavin in its oxidized state (Fl^{ox}) accomplishes amine or amino acid dehydrogenation, the reduced flavin (FlH_2) being reoxidized to Fl^{ox} in a subsequent step at the expense of reduction of O_2 to H_2O_2. Most mechanistic work has been carried out for MAO, where evidence has been obtained for a sequential $e^-/H^+/e^-$ transfer mechanism,[3] as is discussed for heme enzymes[10] and the electrochemical (anodic) oxidation of amines.[11] This reaction has been modeled photochemically,[12] but has not been observed for thermal reactions, and it remains to be determined how the enzyme permits one-electron oxidation to be performed by the singlet flavin cofactor.

Mechanisms for flavin-dependent amine and amino acid oxidations other than SET have been considered, in particular a hydrogen-atom transfer mechanism,[13,4c] a C^{4a} addition-elimination mechanism,[7a,14,15] and, at least in the case of D-amino acid oxidase[8] and sarcosine oxidase,[9] a hydride-transfer mechanism. Recently a modified addition–elimination mechanism has been proposed for MAO-A, involving amine addition to C^{4a} amine concerted with transfer of the amine C_α-proton to N^5.[2d] It is even conceivable that for any given enzyme, the enzyme mechanism may self-tune to the reactivity characteristics of particular substrates. For example, for the MAO inhibitor, *trans*-2-phenylcyclopropylamine,[16] one-electron oxidation is facilitated by coupling to concomitant cyclopropane ring fission,[17] and thus a SET mechanism may be quite favorable in this case. Overall, since no intermediates have been detected during substrate reduction of oxidized flavin, as least in the case of MAO,[18,2d] mechanistic conclusions remain somewhat tentative.

Because ground-state flavins in solution have poor reactivity with amines, various mechanisms have been considered for increasing thermal flavin reactivity. One approach has been the use of models where either hydrogen-bonding and/or π-stacking interactions[19] or stabilization of the bent conformation of the dihydroflavin[20] increase the effective redox potential of the flavin. Another approach is to use so-called 'high potential' flavin analogs with electron-withdrawing substituents or increased delocalization potential.[21] Using one such highly activated flavin, N^5-ethylisoalloxazinium,[22] Mariano and coworkers demonstrated reaction with primary and secondary amines to generate C^{4a} adducts, which could be thermally induced to undergo elimination of imine product in the presence of base (Scheme 1).[14,15]

In order to avoid the bias toward the formation of metastable C^{4a} adducts, we chose to utilize N^1,N^{10}-ethyleneisoalloxazinium (**1**) and analogs with E^o-raising electron-withdrawing 8-Cl or 7-CF_3 (**2**) substituents, as described in the preceding

Keywords: flavin analogs; amine oxidation; monoamine oxidase; addition–elimination.

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Abbreviations: Fl^{ox}, oxidized flavin; MAO, monoamine oxidase; SET, single electron transfer.

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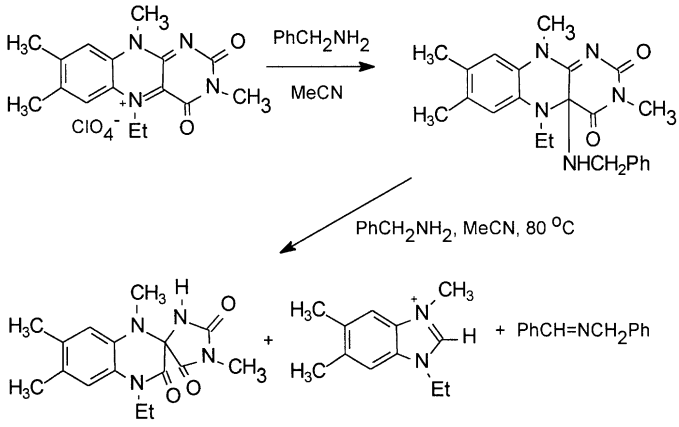
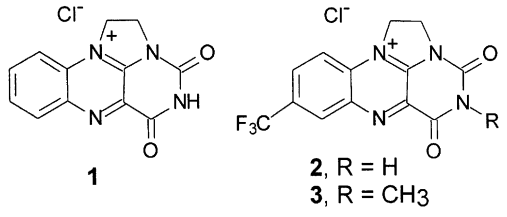


Scheme 1.

paper.[23] The N^3-methyl analog with the 7-CF_3 substituent (**3**) was also prepared to evaluate the effect of blocking reversible N^3-deprotonation in reactions with basic substrates. These compounds were characterized spectroscopically and electrochemically and were shown to react with phenylhydrazine, thiols, and amines. The parent analog **1** was found capable of turnover aerobic oxidation of phenylhydrazine (giving benzene). In the case of amines, the relative reactivity order was found to be primary>secondary>tertiary, opposite to the order seen for oxidation of the same amines by $(phen)_3Fe(III)$. Also, the secondary amines N-methyl and N-cyclopropylbenzylamine were found to yield isolable imine products.

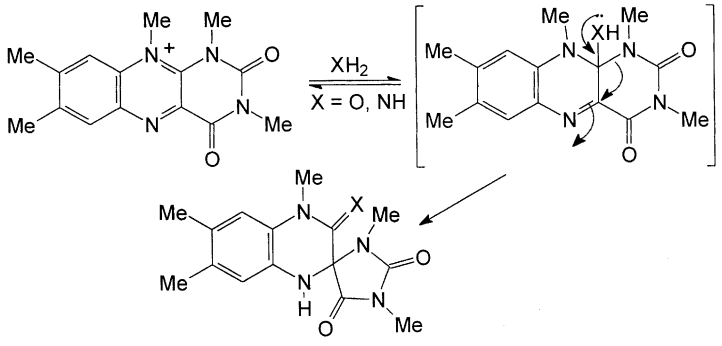


The present study focuses on the use of ^{13}C-labeled versions of the 7-CF_3 flavin **2**, with confirmation using ^{15}N-labeled amine, to elucidate by NMR the nature of intermediates in the reaction with amines, thereby permitting a discussion of mechanisms involved, including that responsible for dehydrogenation of benzylic amines. We find that N^1,N^{10}-dialkylation, like N^5-alkylation, also predisposes the flavin toward an addition–elimination mechanism, but via a C^{10a} as opposed to a C^{4a} adduct. Nonetheless, this work constitutes the first NMR spectroscopic evidence for C^{10a} tetrahedral amino adducts. We further show that whereas tertiary amines themselves fail to generate observable adducts, they induce addition of trace water in the system, leading to spectroscopically (NMR) observable C^{10a} hydroxy adducts.

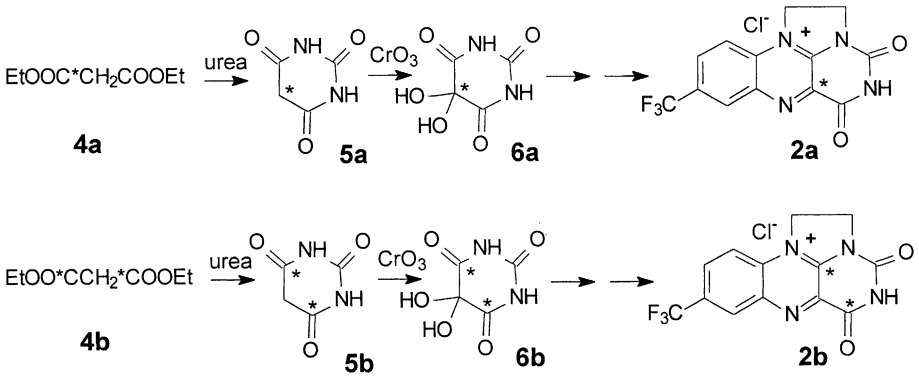
2. Results and discussion

2.1. Reaction of amines with 7-trifluoromethyl-N^1,N^{10}-ethyleneisoalloxazinium chloride (7-CF_3-Fl^+Cl^-, 2)

The flavinium salt **2** is soluble in CF_3COOH-CD_3COOH (6:1), but has very limited solubility in CD_3CN. However, upon the addition of at least one equivalent of secondary or especially primary amine at 25°C, **2** undergoes rapid dissolution in CD_3CN concomitant with appearance of a metastable intermediate in the ^1H NMR spectrum believed to represent an amine–flavin adduct and a variable amount of a minor species containing only flavin signals. The intermediate flavin–amine adduct eventually transforms to a more stable flavin–amine adduct alongside appearance of amine dehydrogenation product (for the benzylic amines). Tertiary amines solubilize only a small amount of **2**, and the ^1H NMR spectra display no evidence of an amine–flavin adduct, instead exhibiting signals only for the



Scheme 2.



Scheme 3.

minor flavin species also seen with primary and secondary amines.

An illustrative example is given for benzylamine. Upon mixing **2** (20 mM) with two equivalents of benzylamine in CD_3CN at 25°C in an NMR tube, the first ^1H NMR spectrum taken (3 min) indicated complete addition of one equivalent of the benzylamine to the flavin nucleus, as indicated by the appearance of two distinguishable diastereotopic benzylic proton signals at 3.38 and 3.43 ppm. The four ethylene-bridge protons appeared at 3.75–3.95 and 4.02–4.11 ppm, with an integral ratio of 3:1, respectively, indicating desymmetrization of the molecule. The signals exhibited by this initial adduct were slowly replaced by a second benzylamine-derived adduct also displaying diastereotopic benzylic protons, with a time constant such that a 1:1 mixture was reached in 5 h. This slow conversion was additionally accompanied by the appearance of signals characteristic of $PhCH_2N{=}CHPh$, resulting from trapping of the benzylamine dehydrogenation product by the second equivalent of benzylamine. Integration of the NMR spectrum over time (using the solvent peak as the common reference) indicated that the $PhCH_2N{=}CHPh$ formed corresponded to nearly one-half (0.49) of the molar amount of initial adduct lost, suggesting that the initial adduct partitions about equally between the second flavin–amine adduct and dihydroflavin (see below) alongside formation of $PhCH_2N{=}CHPh$.

A large body of experimental studies on flavins has revealed the importance of the N-1, C-4a, C-10a and N-5 positions as potential electrophilic centers.[21,24–26] In addition to evidence for C^{4a} adducts arising from N^5-alkylflavinium salts described above, C^{10a} adducts have been implicated in reactions of 1,10-dimethylisoalloxazinium salts with nucleophiles, leading to C^{4a} spirohydantoin rearrangement products,[24,25] as illustrated in Scheme 2. We thus suspected that the initial adducts seen in the reaction of **2** with primary and secondary amines were in fact C^{10a} adducts, and that the second adducts seen represented conversion of the C^{10a} amine adducts to C^{4a} spirohydantoin amidines (X=NR). However, the complexity of the ^1H NMR spectra for these reactions disallowed making unambiguous structural assignments, and we resorted to ^{13}C NMR studies on ^{13}C-enriched analogs of **2**.

2.2. Synthesis of ^{13}C-labeled 7-trifluoromethyl-N^1,N^{10}-ethyleneisoalloxazinium chloride: [4a-^{13}C]7-CF_3-Fl^+Cl^- (2a) and [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (2b)

Two different analogs were prepared (Scheme 3) with an enrichment of 10% ^{13}C either at position 4a or at positions 4 and 10a. The ~10-fold signal enhancement for the labeled positions had the most impact for the normally weak quaternary ^{13}C signals. [5-^{13}C]Barbituric acid (**5a**) was prepared by the condensation of commercially available [2-^{13}C]diethyl malonate with urea in the presence of sodium ethoxide, and was then oxidized with chromic trioxide to the [5-^{13}C]alloxan monohydrate (**6a**), in turn transformed to [4a-^{13}C]7-CF_3-Fl^+Cl^- (**2a**) as in the preceding paper.[23] The [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (**2b**) skeleton was synthesized analogously, starting from the commercially available [1, 3-$^{13}C_2$]diethyl malonate (**4b**). The ^{13}C NMR spectra of **2a** and **2b** in CD_3COOD-CF_3COOH (1:6, v/v) indicated appearance of the C^{4a} signal at 134.3 ppm, and of the C^4 and C^{10a} signals at 158.8 and 145.3 ppm, respectively.

2.3. Reaction of [4a-^{13}C]7-CF_3-Fl^+Cl^- (2a) and [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (2b) with benzylamine

The concentration of **2** used in the reactions with amines followed by ^1H NMR (20 mM) was too low to permit analysis by ^{13}C NMR, and its limited solubility precluded its use at higher concentration. We thus resorted to a protocol where the reactions of **2a/2b** were initially set up at 20 mM in CH_3CN on a 6.0 mL scale. Then, following rapid filtration of some $PhCH_2NH_2HCl$ that precipitated, and concentration at <25°C, the residue was taken up into 0.6 mL of DMSO-d_6 (resulting in a final concentration of 0.2 M) and analyzed by ^{13}C NMR in this solvent.

Assuming addition of amine had occurred at the C^{4a} or C^{10a} position, a large upfield shift of the chemical shift for the respective carbon was expected compared to the starting material, on account of the change in carbon hybridization from sp^2 to sp^3. If the metastable C^{4a} or C^{10a} tetrahedral adducts subsequently underwent elimination of imine, we would expect restoration of sp^2 hybridization (downfield shift) of whatever carbon had initially shifted upfield. The regiochemistry would be clearly demonstrated

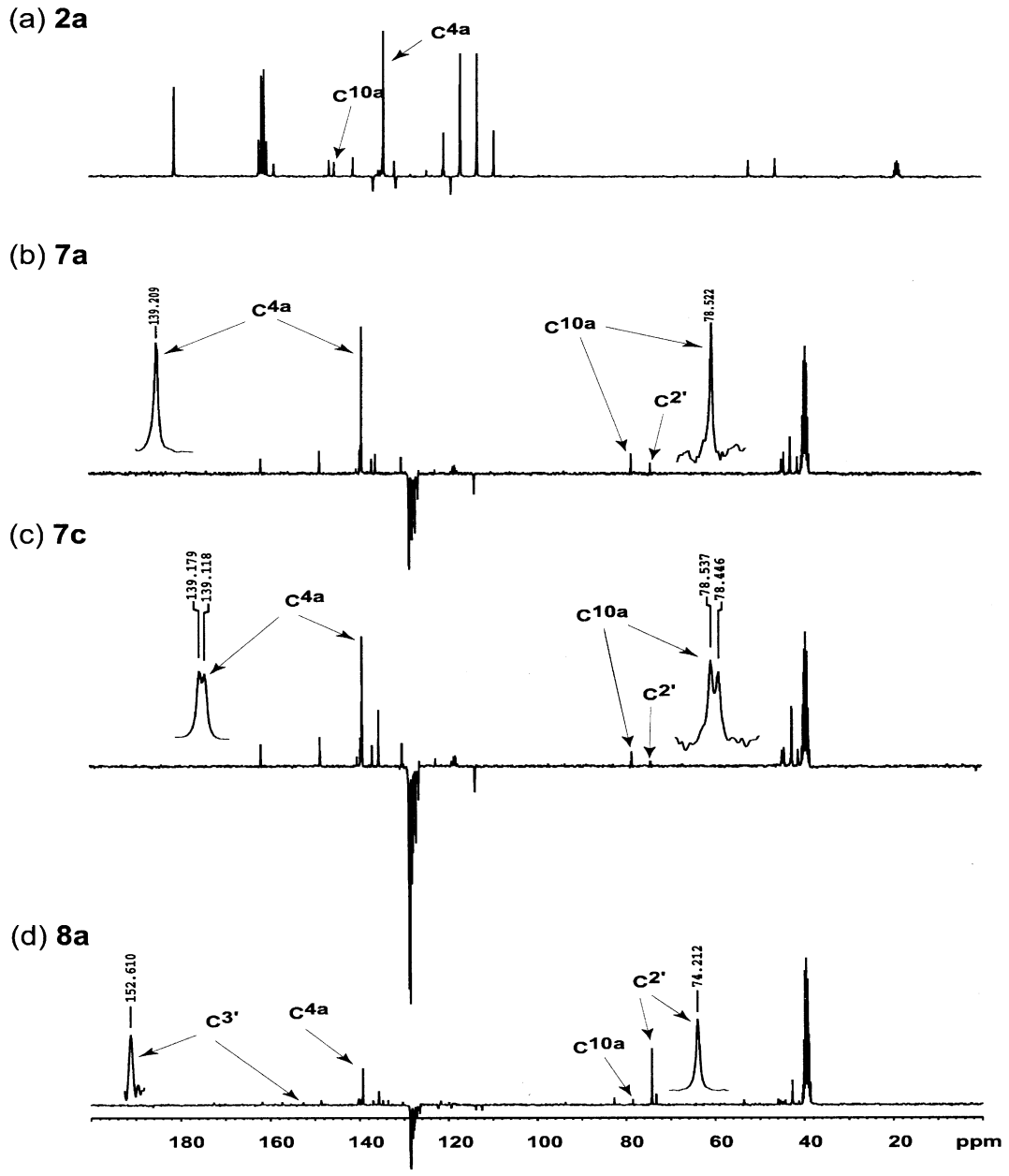


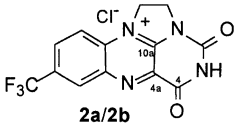
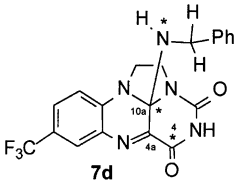
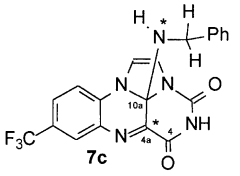
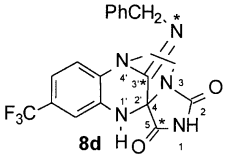
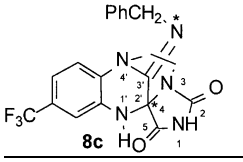
Figure 1. ^{13}C NMR spectra of (a) 200 mM [4a-^{13}C]7-CF_3-Fl^+Cl^- (**2a**) in CD_3COOD/CF_3COOH (1:6), (b) its benzylamine adduct (**7a**) in DMSO-d_6 (200 mM, derived from 20 mM **2a**/40 mM benzylamine in 6 mL of CH_3CN, following filtration, concentration, and dissolution in 0.6 mL of DMSO-d_6 at 25°C), (c) its ^{15}N-benzylamine adduct (**7c**, conditions as for **7a** except using ^{15}N-benzylamine), and (d) the spirohydantoin amidine **8a**, 10 min after heating the sample in spectrum b (**7a**) to 70°C for 10 min.

using the two different ^{13}C-enriched flavin analogs **2a** and **2b**.

In the reaction of benzylamine with [4a-^{13}C]7-CF_3-Fl^+Cl^- (**2a**) the chemical shift of the ^{13}C-labeled 4a position was changed little between the starting flavin (δ 134.3) (Fig. 1a) and the initial benzylamine adduct (δ 139.1) (Fig. 1b), indicating that the hybridization of the **4a** position was unchanged, and thus the amine could not be adding to this position**.** Nonetheless, simultaneous appearance of an upfield absorption at 78.5 ppm (unlabeled, Fig. 1b) indicated some type of tetrahedral benzylamine adduct. This adduct slowly was transformed to the more stable adduct characterized by replacement of the unlabeled 78.5 ppm signal with one at 153.1 ppm alongside an upfield shift of the ^{13}C-label from 139.1 to 74.3 ppm (Fig. 1d) (Table 1). When this latter transformation was accelerated by raising the temperature of the NMR probe 70°C for 10 min, a precursor–product relationship could be established for the observed spectral change by observing the same time constant for the decrease in ^{13}C label at 139.1 and increase at 74.3 ppm.

In the reaction of benzylamine with [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (**2b**) the rapid adduct formation was accompanied by a shift of the ^{13}C-labeled signals of the starting material at 145.3 and 158.8 ppm (Fig. 2a) to 78.5 and 161.8 ppm (Fig. 2b), and a change of the two-bond

Table 1. ^{13}C NMR chemical shifts and $^{15}N^{13}C/^{13}C^{13}C$ coupling constants for adducts formed in the reaction of [^{15}N[benzylamine with the ^{13}C-labeled 7-(trifluoromethyl)-N^1,N^{10}-ethyleneisoalloxazinium chlorides **2a/2b** in DMSO-d_6

		C^{10a}	C^{4a}	C^4	PhCH_2-NH-	$^2J(C^{10a}$-$C^4)$
 **2a/2b**	δ *J*	145.3[a]	134.3[b]	158.8[a]		 10.1[a]
 **7d**	δ *J*	78.5[c] 6.6 ($^1J[^{13}C,^{15}N]$)	139.2	161.8[c]	44.3 4.6 ($^1J[^{13}C,^{15}N]$)	8.6
 **7c**	δ *J*	78.5 6.8 ($^1J[^{13}C,^{15}N]$)	139.1[c] 4.5 ($^2J[^{13}C,^{15}N]$)	161.8	44.3 4.3 ($^1J[^{13}C,^{15}N]$)	
		$C^{3'}$	$C^{2'}$	C^5	PhCH_2-NH-	$^2J(C^{3'}$-$C^5)$
 **8d**	δ *J*	152.8[c] 2.4 ($^1J[^{13}C,^{15}N]$)	74.2 6.1 ($^2J[^{13}C,^{15}N]$)	173.2[c]	45.9 4.6 (^{1}J[^{13}C,^{15}N])	none
 **8c**	δ *J*	153.1	74.3[c] 6.0 ($^2J[^{13}C,^{15}N]$)	173.6	45.8 4.5 ($^1J[^{13}C,^{15}N]$)	

[a] From the NMR spectrum of compound **2b** in CD_3COOD/CF_3COOH (1:6).
[b] From the NMR spectrum of compound **2a** in CD_3COOD/CF_3COOH (1:6).
[c] Selectively enhanced signal.

^{13}C-^{13}C coupling from 10.1 Hz in the starting flavin **2b** to 8.5 Hz in the adduct (Table 1). Maintenance of the latter coupling indicated that the alloxan-derived ring was intact. We assign the most downfield signal in both cases as the C^4 carbonyl, thus indicating that the 78.5 ppm signal, presumed above to be associated with generation of a tetrahedral benzylamine adduct, represents C^{10a}. We thus assign the initial benzylamine adduct as C^{10a} adduct **7** (Scheme 4). It should be noted that we have omitted in Scheme 4 the competing dehydrogenation reaction forming PhCH=NCH_2Ph for the sake of clarity at this point.

After documenting formation of the C^{10a} adduct, it seemed clear that the more stable rearranged adduct was probably spirohydantoin amidine **8** (Scheme 4), as expected in analogy to the reaction of N^1,N^3,7,8,N^{10}-pentamethylisoalloxazinium with either aqueous base or ethanolic NH_3 shown above in Scheme 2.[24] Although such rearrangement has not been reported for N^1,N^{10}-ethyleneisoalloxazinium salts, and it was not obvious that it would be sterically allowed, molecular modeling (see below) revealed no steric impediment to forming 3′,4′-dihydro-3′-benzylimino-3,4′-ethylene-7′-(trifluoromethyl)spiro[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (**8**).

2.4. Reaction of [4a-^{13}C]7-CF_3-Fl$^+$Cl$^-$ (2a) and [4,10a-$^{13}C_2$]7-CF_3-Fl$^+$Cl$^-$ (2b) with [^{15}N]-benzylamine

Further confirmation for the structure of the C^{10a} tetrahedral benzylamine adduct **7** and subsequently formed spirohydantoin amidine **8** came from ^{13}C NMR spectral investigation of the reaction of the two ^{13}C-enriched flavins with ^{15}N-enriched benzylamine. Using [4a-^{13}C]7-CF_3-Fl$^+$Cl$^-$ (**2a**), in contrast to what was seen with unlabeled benzylamine (Fig. 1b), the spectrum of the adduct **7c** obtained with [^{15}N]-benzylamine (Fig. 1c) exhibited splitting of the ^{13}C-enriched C^{4a} signal at 139.1 ppm into a doublet with a 4.5 Hz coupling. The unlabeled ^{13}C signal at 78.5 ppm was shown also to be a doublet with a coupling constant of 6.8 Hz. In the case of [4,10a-$^{13}C_2$]7-CF_3-Fl$^+$Cl$^-$ (**2b**), the now ^{13}C-enriched C^{10a} signal at 78.5 ppm appeared as a triplet, owing to splitting by ^{15}N with a coupling constant on the same order as that of the C^{10a}-C^4 ^2J coupling (8.5 Hz) (Fig. 2c). Our interpretation of this data is that the larger ^{13}C-^{15}N coupling seen for the upfield ^{13}C signal (78.5 ppm) in the benzylamine adduct represents the ^1J coupling between ^{15}N and $^{13}C^{10a}$ in the C^{10a} benzylamine adduct **7**, whereas the smaller ^{13}C-^{15}N coupling seen for the downfield ^{13}C signal (139.1 ppm) represents the ^2J $^{13}C^{4a}$-^{15}N coupling. All the spectral assignments are summarized in Table 1.

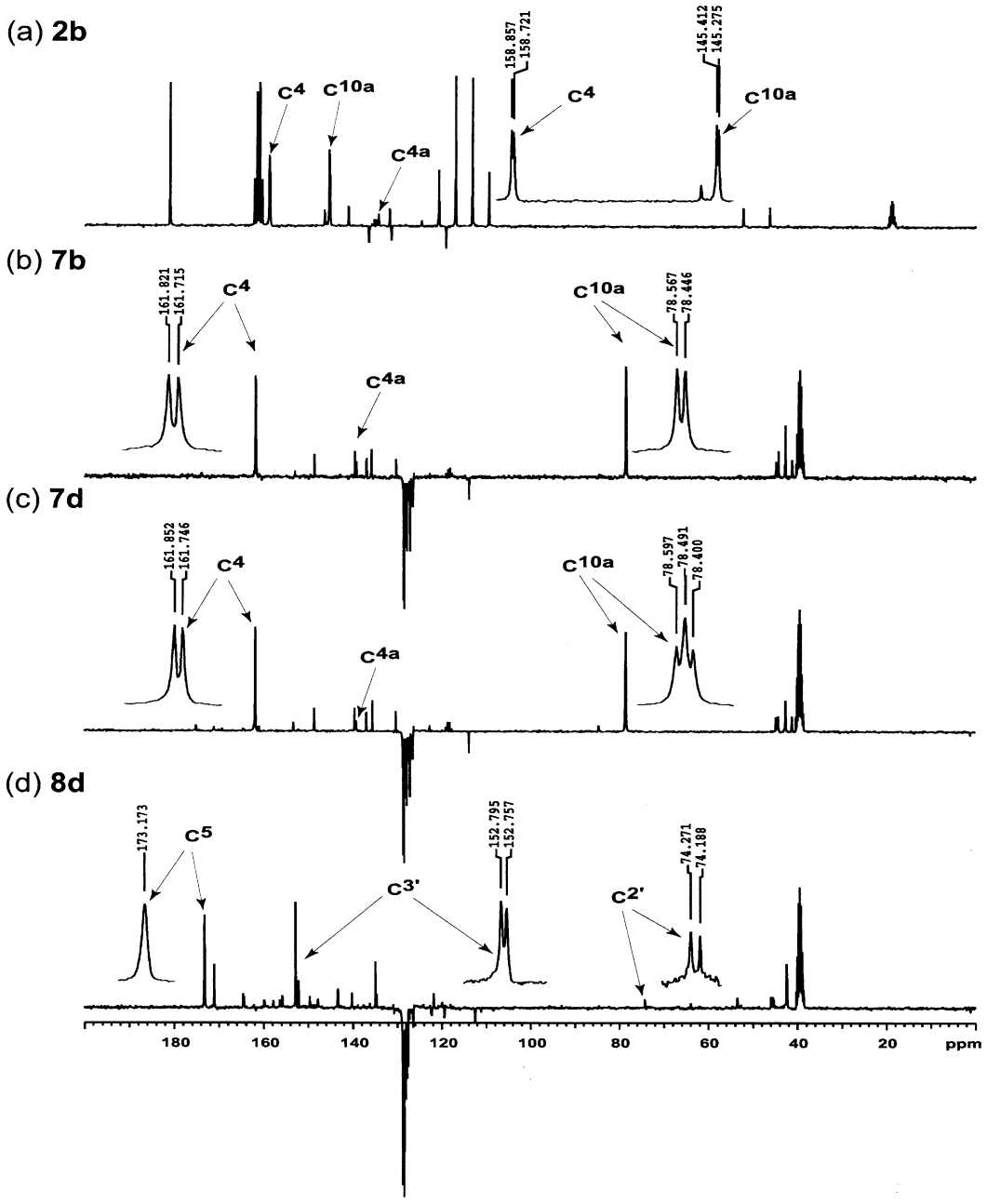
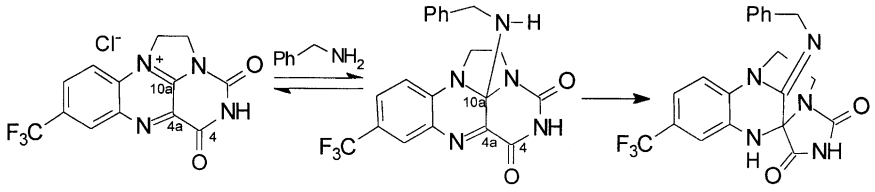


Figure 2. ^{13}C NMR spectra of (a) 200 mM [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (**2b**) in CD_3COOD/CF_3COOH (1:6), (b) its benzylamine adduct (**7b**) in DMSO-d_6 (200 mM, derived from 20 mM **2b**/40 mM benzylamine in 6 mL of CH_3CN, following filtration, concentration, and dissolution in 0.6 mL of DMSO-d_6 at 25°C), (c) its ^{15}N-benzylamine adduct (**7d**, conditions as for **7b** except using ^{15}N-benzylamine), and (d) the spirohydantoin amidine **8d**, 10 min after heating the sample in spectrum c (**7d**) to 70°C for 10 min.

Upon raising the temperature of the NMR probe to 70°C for 10 min in order to accelerate rearrangement of the C^{10a} benzylamine adduct **7** to spirohydantoin amidine **8**, the fate of the ^{13}C-enriched C^{10a} signal at 78.5 ppm (using **2b**) could be traced to a signal at 152.8 ppm assigned to $C^{3'}$ of the spirohydantoin amidine **8d** (Fig. 2d), reflecting rehybridization of this carbon from sp^3 to sp^2. At the same time, the ^{13}C-enriched C^{4a} signal at 161.8 ppm (using **2a**) could be traced to a signal at 74.3 ppm assigned to $C^{2'}$ of the spirohydantoin amidine (see **8a** in Fig. 1d), reflecting rehybridization of this carbon from sp^2 to sp^3. The ^{13}C-enriched C^4 signal at 161.8 of the initial adduct (using **2b**) could be traced to a signal at 173.2 assigned to C^5 of the spirohydantoin amidine **8d** (Fig. 2d). Confirmation of the ^{13}C-enriched signals at 152.8 and 173.2 as representing $C^{3'}$ and C^5 of the spirohydantoin amidine, respectively, was that the former signal exhibited coupling to ^{15}N (2.4 Hz, consistent with a doubly-bonded ^{13}C-^{15}N ^1J),[27–29] whereas the latter signal showed no splitting by ^{15}N (Fig. 2d). In addition, the ^{13}C signal for $C^{2'}$ at 74.3 ppm exhibited two-bond coupling to ^{15}N (6.0 Hz). Interestingly, whereas two-bond coupling between C^{10a} and C^4 was clearly evident in the initial benzylamine adduct **7b** (Fig. 2b) and **7d** (Fig. 2c), this coupling disappeared upon rearrangement to spirohydantoin amidine **8b** and **8d** (Fig. 2d), even though these carbons ($C^{3'}$ and C^5) retain their two-bond connectivity. It



2 (without ^{13}C label)
2a ([4a-^{13}C]7-CF_3-Fl$^+Cl^-$)
2b ([4,10a-$^{13}C_2$]7-CF_3-Fl$^+Cl^-$)

7 (without ^{13}C label)
7a ([4a-^{13}C 7-CF_3-Fl$^+Cl^-$/ benzylamine)
7b ([4,10a-$^{13}C_2$]7-CF_3-Fl$^+Cl^-$/ benzylamine)
7c ([4a-^{13}C]7-CF_3-Fl$^+Cl^-$/ [^{15}N]-benzylamine)
7d ([4,10a-$^{13}C_2$]7-CF_3-Fl$^+Cl^-$/ [^{15}N]-benzylamine)

8 (without ^{13}C label)
8a ([4a-^{13}C]7-CF_3-Fl$^+Cl^-$/ benzylamine)
8b ([4,10a-$^{13}C_2$]7-CF_3-Fl$^+Cl^-$/ benzylamine)
8c ([4a-^{13}C]7-CF_3-Fl$^+Cl^-$/ [^{15}N]-benzylamine)
8d ([4,10a-$^{13}C_2$]7-CF_3-Fl$^+Cl^-$/ [^{15}N]-benzylamine)

Scheme 4.

appears that the conformational relationship of the two sp^2 carbons connected through the tetrahedral spiro center results in loss of spin–spin coupling.

We have depicted spirohydantoin amidine **8** in the *Z* configuration (benzyl group *cis* to $N^{4'}$). The availability of ^{13}C-^{15}N coupling constants led us to consider whether these values might provide evidence for the actual configuration, since it is known that the magnitude of the two-bond ^{15}N-^{13}C coupling in C=N-containing compounds depends on whether the coupled C is *syn* or *anti* to the N sp^2 lone pair. In a number of oximes, values of ^2J in the range of 0.3–1.8 Hz have been observed for ^{13}C *anti* to the N lone pair, whereas values of 9.2–11.6 Hz have been observed for ^{13}C *syn* to the N lone pair.[27–29] Our value of 6.0–6.1 Hz for the ^2J ^{13}C-^{15}N coupling in **8c/8d** is somewhat closer to the higher rather than lower range, consistent with our depiction. Independent evidence for the amidine configuration was obtained utilizing a molecular mechanics computation (see Experimental Section). In the final energy-minimized structure of **8**, the nitrogen lone-pair is in a *syn* orientation relative to the coupled carbon ($C^{2'}$), and the dihedral angle (N=$C^{3'}$-$C^{2'}$-C^5) is 9.47° (Fig. 3).

2.5. Direct isolation of C^{4a} spirohydantoin amidines

Our proposal that the metastable flavin–amine adduct seen by NMR represents the C^{10a} addition product **7** is important in that this represents a prototypical 'tetrahedral intermediate' (sp^3 carbon bonded to three heteroatoms), which has rarely been observed directly. Consistent with the expectations of such an intermediate, attempted isolation

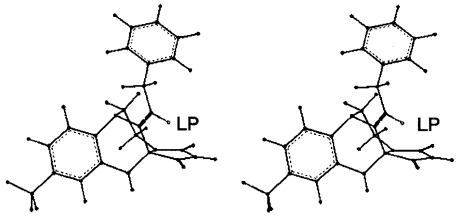
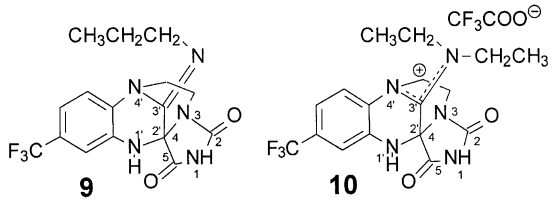


Figure 3. Stereoview of the minimum energy structure for spirohydantoin amidine **8** (depicting the location of nitrogen lone-pair (**LP**)), using the program 'Discover' with the CFF91 forcefield.

of **7** resulted in recovery of the starting oxidized flavin **2**. Thus, confirmation of the NMR-based structural assignments made above was by isolation and characterization of the C^{4a} spirohydantoin amidines. Spirohydantoin amidine **8** derived from benzylamine was unstable to isolation, presumably due to the lability of the N-benzyl moiety. In contrast, the use of propylamine and diethylamine rather than benzylamine permitted the isolation of the more stable spirohydantoin amidines 3′,4′-dihydro-3′-propylimino-3,4′-ethylene-7′-(trifluoromethyl)spiro[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (**9**) and 3′,4′-dihydro-3′-diethylimino-3,4′-ethylene-7′-(trifluoromethyl)spiro[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (**10**), and their characterization by ^1H NMR, ^{13}C NMR, and HRMS. Structural characterization of these spiro compounds serves as presumptive evidence for the structure of the tetrahedral intermediates we claim to be C^{10a} flavin–amine adducts.

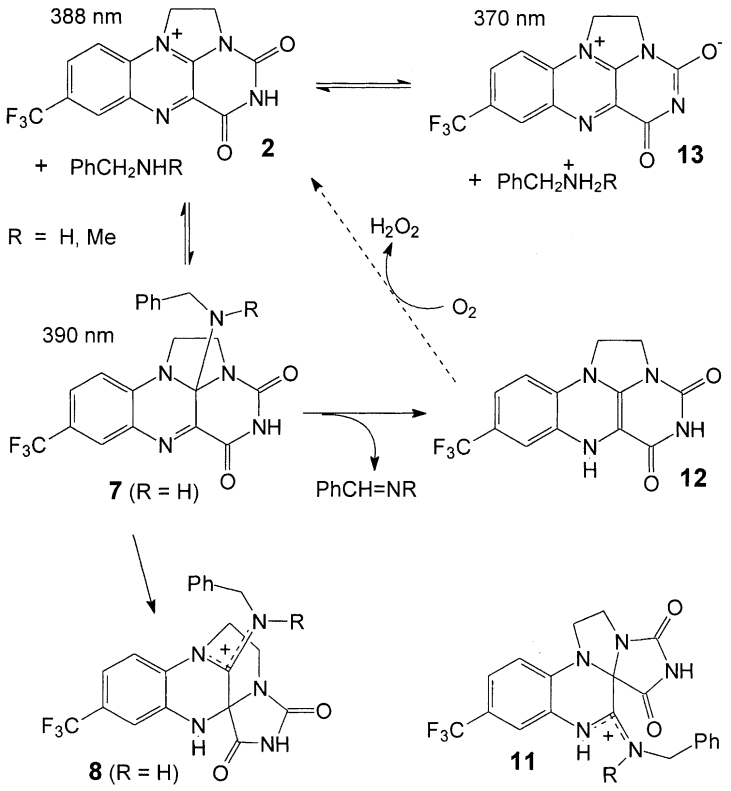


9 **10**

2.6. Proposed addition–elimination mechanism for amine oxidation

As described in the preceding paper,[23] reactions of **2** (or **3**) with benzylamine in CH_3CN-buffer using periodic admission of O_2 to allow for reoxidative cycling of the reduced flavin generated upon oxidation of benzylamine to benzaldehyde, revealed a modest level of turnover (the yield of benzaldehyde was more than 100% based on flavin), suggesting that at least some of the reaction proceeds through generation of the reduced flavin **12** (Scheme 5), which is reoxidized to starting **2** (or **3**).

Nonetheless, the observed plateauing of the benzaldehyde yield can now be understood in terms of irreversible removal of the catalytic flavin from the system by its eventual complete channeling to spirohydantoin amidine **8**. The observation of PhCH=NCH_2Ph also in the above-described



Scheme 5.

reactions followed by NMR in CD_3CN (^1H NMR) or DMSO-d_6 (^{13}C NMR) also supports the notion of a partitioning of the initial C^{10a} adduct between β-elimination to give dehydrogenation product and rearrangement to give spirohydantoin amidine (Scheme 5). The generation of reduced flavin was verified in the case of the reactions of **2** in CD_3CN with benzylamine (giving $PhCH{=}NCH_2Ph$), N-methylbenzylamine (giving $PhCH{=}NCH_3$), and N-cyclopropylbenzylamine (giving PhCH=N-*c*-C_3H_5) through the isolation of the N^5-acetyl derivative of **12** following Ac_2O quenching of the reaction under anaerobic conditions.

For the reaction of **2** with the unactivated amines propylamine and diethylamine described above, the resulting spirohydantoins **9** and **10** were stable and could be isolated from the reaction, but no significant levels of dehydrogenation products were detected in these cases. According to Scheme 5, the partitioning of the initially formed C^{10a} adduct between β-elimination and rearrangement (giving spirohydantoin amidine) should depend sensitively on whether the adducted amine is activated toward dehydrogenation. Thus, in contrast to the case of benzylic amines, dehydrogenation for the non-benzylic primary and secondary amines is apparently too slow to compete with the rearrangement reaction in these cases.

This profile is analogous to that concluded by Mariano and coworkers using an N^5-ethyl flavinium salt, where the amine C^{4a} adducts partition between β-elimination and two 'decomposition' reactions, one being rearrangement to a C^{10a} spirohydantoin amidine.[14] Theoretically, we cannot rule out the possibility that the dehydrogenation products seen in our reactions also arise from a C^{4a} adduct which, in our case, would represent a minor ('invisible') side-equilibrium in competition with the C^{10a} adduct. Draining off of a minor C^{4a} adduct toward dehydrogenation would just shift the equilibrium and give the (false) appearance of a precursor-product relationship between C^{10a} adduct and dehydrogenation. However, *if the C^{4a} adduct were generated from **2**, we should have seen at least some of the side-products observed for C^{4a} adducts*,[14] such as the corresponding C^{10a} spirohydantoin amidine **11** (Scheme 5). The spirohydantoin amidine formed in our case is clearly not **11**, because if it were, then in the reaction of amine with [4a-^{13}C]7-CF_3-Fl^+Cl^- (**2a**), a large downfield shift of the labeled ^{13}C would have been observed, in contrast to the large upfield shift seen. As we saw no evidence of any side-products expected from a C^{4a} adduct, we feel confident that the dehydrogenation products observed from the benzylic amines stem from C^{10a} rather than C^{4a} adducts.

Included in Scheme 5 is a side-equilibrium depicting reversible deprotonation of flavin **2** by amine in competition with addition. This reaction was revealed by spectroscopic studies on the reaction of **2** at low concentration (0.05 mM) with excess amines in CH_3CN-buffer,[23] wherein the Fl^{ox} absorption spectrum was immediately replaced by one exhibiting a λ_{max} at 370 nm regardless of whether the amine was 1°, 2°, or 3°. Since no spectral change was seen using the 3-methyl 7-CF_3-flavin analog **3**, we interpreted the 370 nm absorption to represent the equilibrium presence of the zwitterion **13** generated upon N^3-deprotonation of **2**. In the case of *primary and secondary* amines, the 370 nm absorption subsequently transposed to a 390 nm absorption

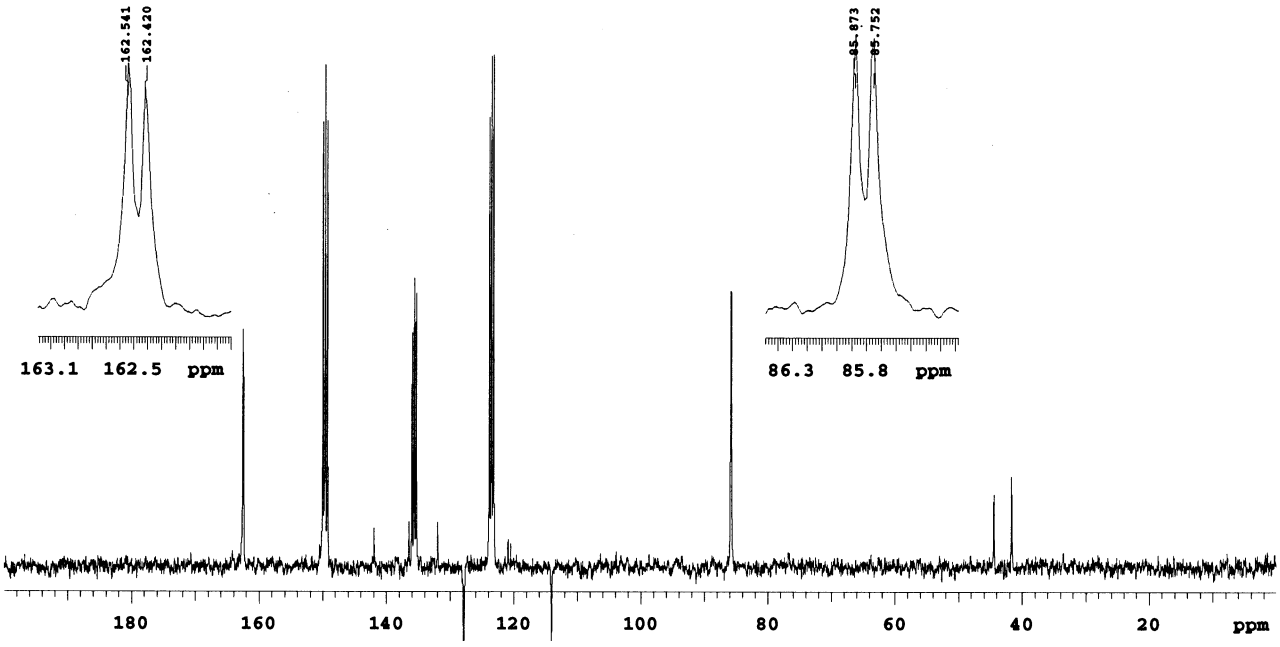


Figure 4. ^{13}C NMR spectrum (pyridine-d_5) of the C^{10a} hydroxy adduct **14**, enriched with 10 atom% ^{13}C at positions 4 and 10a (derived from **2b**).

that we interpreted to represent an amine adduct of **2**, now being identified as the C^{10a} adduct **7**. The finding that the decrease in A_{370} was greater for primary than for secondary amines[23] can now be interpreted to imply that primary amines form C^{10a} adducts more rapidly than secondary amines, as expected on steric grounds. In the case of **3**, the starting flavin absorption (388 nm) was directly transposed to the 390 nm absorption, more rapidly with primary than secondary amines (data not shown). Although one might have expected N^3-deprotonation to have resulted in a longer wavelength absorption than C^{10a} amine addition, zwitterion **13** is cross-conjugated and is evidently less delocalized than the starting flavinium cation (388 nm). Further evidence that the 390 nm absorption truly corresponds to the C^{10a} adduct **7** seen by NMR was obtained by diluting into CH_3CN an aliquot of the NMR tube reaction of **2** with benzylamine at the point of maximum appearance of **7**; the immediately recorded UV spectrum showed the 390 nm band.

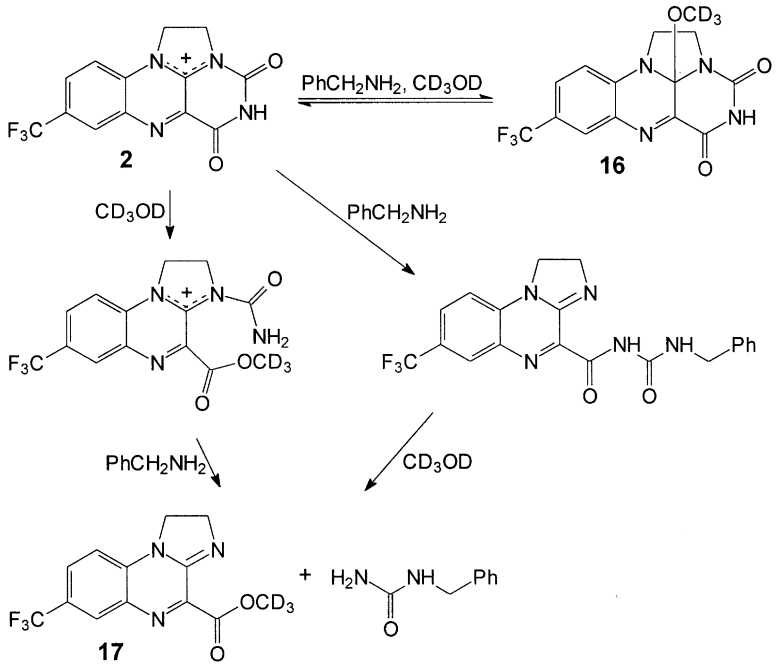
Whether generated from **2** or **3**, the subsequent slow decay of the 390 nm absorption was interpreted to represent the irreversible reaction(s) occurring between **2/3** and amines,[23] now shown for **2** to represent the conversion of **7** to the spirohydantoin **8** and, in the case of benzylic amines, additionally to reduced flavin and dehydrogenation product.

Despite the clear appearance of what we describe as zwitterion **13** when flavin **2** and amines are first mixed at low concentration in CH_3CN,[23] no evidence for this species was obtained from the NMR studies described above. Our explanation for this is that at the much higher concentration needed for NMR characterization, complete formation of C^{10a} amine adduct **7** had occurred by the time the first spectrum could be obtained. In the case of *tertiary* amines, where no amine adduct is generated, the zwitterion **13** was still not seen by NMR, for the reason revealed in the next section.

2.7. NMR Spectroscopic characterization of a flavinium C^{10a} tetrahedral hydroxy adduct

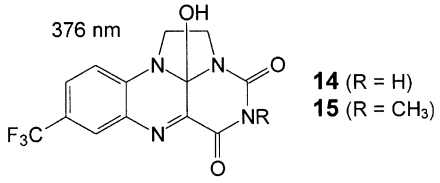
In our studies involving UV-visible monitoring of the reaction of **2** with *tertiary* amines,[23] the initially observed absorption at 370 nm (due to N^3-deprotonation) was transposed to a band at 376 nm instead of 390 nm observed for primary and secondary amines. Also, in the reaction of tertiary amines with the N^3-methyl flavin **3**, the oxidized flavin bands were replaced directly by the 376 nm band. Whereas the 390 nm band has now been identified to represent C^{10a} amine adducts, it did not appear that the 376 nm band could be ascribed to a tertiary amine C^{10a} adduct. In fact, the identical pattern of flavin-derived ^1H NMR signals was generated using a variety of tertiary amines (Et_3N, $PhCH_2N(CH_3)_2$ or pyridine) in CD_3CN, which would not be the case for covalent amine adducts. Since a C^{10a} hydroxy adduct has been proposed as an intermediate in the base-induced conversion of N^1,N^{10}-alkylated flavinium salts to C^{4a}-spirohydantoin amides,[24–26] we considered that C^{10a} hydroxy adduct **14** would explain not only the common 376 nm absorption but also the common ^1H NMR spectrum, independent of the identity of the tertiary amine. The CD_3CN solvent used for NMR studies would only have to contain 0.04% water to permit complete conversion of the 20 mM **2** to its C^{10a} hydroxy adduct in the presence of base. Consistent with this interpretation, when **2** was suspended in dry CD_3CN (distilled from CaH_2), addition of tertiary amine did not induce dissolution of **2** and no ^1H NMR spectrum was obtainable.

Verification of the structure of C^{10a} hydroxy adduct **14** came from the ^{13}C NMR spectrum obtained for the reaction of 20 mM ^{13}C-enriched [4,10a-$^{13}C_2$]7-CF_3-Fl^+Cl^- (**2b**) and 2 equivalents of water in 0.6 mL of pyridine-d_5 (Fig. 4). That addition had occurred at the 10a position was clearly shown by a large upfield shift of the $^{13}C^{10a}$ resonance, compared to that of the starting flavin. Further support for the structural



Scheme 6.

assignment of **14** as the C^{10a} hydroxy adduct came from observation of spin-spin coupling between the C^{10a} and C^4 atoms of 8.6 Hz, identical with that seen for the structurally similar primary amine adduct **7**. After the NMR spectrum was recorded (e.g. Fig. 4), a portion of the sample was removed from the NMR tube and diluted 400 fold with dry CH_3CN for UV spectral study; a peak at 376 nm was observed. Consistent with hydroxy adduct **14** giving rise to the 376 nm band, the latter was seen to form more rapidly with increasing amounts of water added.



As discussed above, ^1H NMR spectral monitoring of the reaction of **2** with *primary or secondary* amines in CD_3CN at 25°C revealed an immediate buildup of mainly the C^{10a} amine adducts but also another species containing only flavin signals. This minor species displayed identical NMR signals as those seen for the C^{10a} hydroxy adduct generated from the reaction of **2** and tertiary amines in CD_3CN. Thus, the C^{10a} amine and hydroxy adducts appear to coexist and to not rapidly equilibrate on the NMR time scale. In fact, when N^3-methyl flavin **3**, which gave the analogous C^{10a} hydroxy adduct **15** in wet pyridine-d_5, was exposed to benzylamine in mixtures of CD_3CN and pyridine-d_5, both the C^{10a} amine and hydroxy adducts could be seen clearly during the first 5 min after mixing (not shown), before the onset of C^{10a} adduct decay. The relative proportion of the two adducts varied with solvent composition, the amine adduct being favored in CD_3CN and the hydroxy adduct being favored in pyridine-d_5 (data not shown). Although the nature of this solvent effect was not explored, it seems clear that the two adducts are in slow equilibrium with each other through their common precursor **3**.

2.8. Reaction of 7-CF_3-Fl^+Cl^- (2) with amines in methanol

The reaction of **2** with amines in CH_3CN has been described above to yield initially C^{10a} amine adducts (1° and 2° amines only) in competition with a low level of C^{10a} hydroxy adduct (all amines) when water is present. However, when **2** was treated with *any* amine (1°, 2°, 3°) in CD_3OD, a single new adduct, which we identify as the C^{10a} flavin–methoxy adduct **16** (Scheme 6), formed immediately to the exclusion of C^{10a} amine adducts, and the latter could not be observed at any point in the reaction. The ^1H NMR spectrum of the methoxy adduct of **2** shows the four ethylene bridge protons at 4.04 ppm (m) and 4.42 ppm (m) with an integral ratio 3:1. The ^{13}C NMR spectra of the methoxy adduct and those of the corresponding C^{10a} amine and hydroxy adducts were almost identical. An upfield ^{13}C NMR absorption was detected at 90.6 ppm indicating that some carbon (presumably C^{10a}) was undergoing a hybridization change from sp^2 to sp^3.

Over several hours (depending on the amine used), the spectrum of the C^{10a} methoxy adduct deteriorated, accompanied by formation of stable end products. Using benzylamine, the NMR spectrum after 24 h displayed a stable product identified as methyl ester **17**, which was isolated in deuterated form directly from the NMR tube and characterized by ^1H NMR, ^{13}C NMR and HRMS. A side product, benzylurea, was confirmed by ^1H NMR, ^{13}C NMR, and TLC by comparison with the commercial compound. Although **2** is in equilibrium with the C^{10a} methoxy adduct, unlike the C^{10a} hydroxy and amine adducts, the methoxy adduct cannot decompose to a spirohydantoin (no heteroatom-bound

proton is available). In the absence of an obvious pathway for further transformation of the C^{10a} adduct, we propose that **2** reacts alternately to give the observed end products by attack of benzylamine at the electrophilic C^2 carbonyl and of methanol at the electrophilic C^4 carbonyl (in either order, Scheme 6).

3. Conclusions

In summary, the first direct evidence of a flavin C^{10a} tetrahedral amine adduct has been obtained from NMR studies of the reaction of ^{13}C-labeled N^1,N^{10}-ethylene-bridged flavinium salts with primary and secondary amines, as supported using ^{15}N-labeled benzylamine. Further support came from the isolation and full characterization of C^{4a}-spirohydantoin amidines that derive from the initial C^{10a} adducts, especially in the case of unactivated (e.g., non-benzylic) amines. In the case of benzylic amines, the initial C^{10a} adducts also undergo an approximately equal extent of β-elimination of imine oxidation product. The β-elimination pathway is accompanied by generation of the reduced dihydroflavin (confirmed by Ac_2O trapping), which can be re-oxidized to the starting flavin. However, although the 7-CF_3-substituted flavinium salt featured in this study was most active in amine dehydrogenation, the 7-CF_3 substituent appears to retard aerobic reoxidation of the reduced flavin, so that efficient turnover catalysis is not seen.[23] It appears that the factor limiting turnover achievable in the current system, utilizing a C^{10a} addition–elimination mechanism, is the ultimate irreversible draining off of active catalyst in forming spirohydantoin amidines.

Since the mechanism of amine and amino acid dehydrogenation by flavin-dependent enzymes continues to be a matter of active research, the studies reported here provide important information pertinent to those discussions that consider addition–elimination as a potential enzymologic mechanism.

4. Experimental

4.1. General procedures

[2-^{13}C]Diethyl malonate (99 atm%) and the unlabeled amines were obtained from Aldrich Chemical Co., Inc. [1,3-$^{13}C_2$]Diethyl malonate (99 atom%) and ^{15}N-labeled benzylamine (99 atom%) was purchased from Isotec Inc. and used without further purification. ^1H NMR and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively. Proton chemical shifts (δ) are reported in parts per million (ppm) relative to the residual CHD_2 methyl quintuplets in the deuterated solvents (1.93 ppm acetonitrile-d_3, 3.30 ppm for methanol-d_4, or 2.49 ppm for DMSO-d_6). Carbon chemical shifts are reported in parts per million relative to the internal ^{13}C signals in CD_3CN (117.61 and 0.60 ppm), CD_3OD (49.00 ppm), DMSO-d_6 (39.50 ppm) and pyridine-d_5 (123.5, 135.5 and 149.9 ppm). High-resolution mass spectra (HRMS) were obtained by electron impact ionization (20–40 eV) or fast atom bombardment (FAB). UV–visible spectra were recorded with a Perkin–Elmer model Lambda 3B spectrophotometer fitted with a water-jacketed multiple cell holder for maintenance of constant temperature. Spectral scans were obtained using the PECSS software. 7-(Trifluoromethyl)-1,10-ethylene-isoalloxazinium chloride (**2**) was prepared as described in the preceding paper.[23]

4.2. Preparation of [5-^{13}C]barbituric acid (5a)

To the clear solution resulting from addition of sodium (718 mg, 31.2 mmol) to 16 mL of absolute ethanol under argon was added 10% ^{13}C-enriched [2-^{13}C]diethyl malonate (**4a**) (5.0 g, 31.2 mmol), followed by a solution of dry urea (1.9 g, 31.2 mmol) in 16 mL of hot absolute ethanol (70°C). The solution was heated at 110°C for 7 h. A white solid separated rapidly. The reaction mixture was then treated with 32 mL of hot water (50°C) and then with 4N hydrochloric acid (~13 mL), with stirring, until the solution was acidic. After the mixture was filtered, the resulting almost clear filtrate was stored in the refrigerator overnight. The crystallized material was collected by filtration and washed thoroughly with cold water. After drying in vacuo, 3.5 g (87%) of 10% ^{13}C-enriched [5-^{13}C]barbituric acid (**5a**) was obtained as a bright white powder. ^1H NMR (DMSO-d_6) δ 3.45 (s, 2H), 11.12 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 39.3 (*C-5), 151.6, 167.8. **[4,6-$^{13}C_2$]Barbituric acid (5b).** The synthesis of the 10% ^{13}C-enriched [4,6-$^{13}C_2$]-barbituric acid (**5b**) was accomplished by the same method and began with 10% ^{13}C-enriched [1,3-$^{13}C_2$]diethyl malonate (**4b**). ^1H NMR (DMSO-d_6) δ 3.45 (s, 2H), 11.12 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 39.3, 151.6 (*C-4), 167.8 (*C-6).

4.3. Preparation of [5-^{13}C]alloxan monohydrate (6a)

To a solution of chromium trioxide (4.1 g, 41.3 mmol) in glacial acetic acid (20 mL)/water (2.5 mL) was added **5a** (3.5 g, 27.3 mmol) slowly at 25°C and the reaction mixture was stirred for 30 min at 50°C. During the reaction, alloxan monohydrate began to crystallize. After the slurry was cooled at 5°C the precipitate was filtered, washed with cold glacial acetic acid (40 mL) and then cold Et_2O (10 mL). Removal of final traces of solvent in vacuo left 10% ^{13}C-enriched [5-^{13}C]alloxan monohydrate (**6a**) as a pale yellow powder (2.90 g, 66%). ^1H NMR (DMSO-d_6/$CDCl_3$) δ 7.48 (s, 2H), 11.3 (s, 1H), 11.6 (s, 1H); ^{13}C NMR (DMSO-d_6/$CDCl_3$) δ 84.8 (*C-5, *C(OH)$_2$[hydrate form]), 149.5 (C-2 and C-2′), 156.5 (C-4′ and C-6′), 167.3 (*C-5′, *C=O[keto form]), 168.7 (C-4 and C-6). **[4,6-$^{13}C_2$]Alloxan monohydrate (6b).** The synthesis of the 10% ^{13}C-enriched [4,6-$^{13}C_2$]alloxan monohydrate was accomplished by the same method and began with **5b**. ^1H NMR (DMSO-d_6) δ 7.56 (s, 2H), 11.3 (s, 1H), 11.6 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 84.9 (C-5, C(OH)$_2$[hydrate form]), 149.5 (C-2 and C-2′), 156.5 (*C-4′ and *C-6′), 167.3 (C-5′, C=O[keto form]), 168.9 (*C-4 and *C-6).

4.4. Synthesis of ^{13}C-enriched 7-(trifluoromethyl)-flavinium chlorides 2a and 2b

The two 10% ^{13}C-enriched 7-(trifluoromethyl)flavinium chlorides were synthesized from 4-bromo-3-nitrobenzotrifluoride and the corresponding 10% ^{13}C-enriched alloxan monohydrate in the same manner as described for the

unlabeled compound.[23] Analytical data: **[4a-^{13}C]7-(Trifluoromethyl)-1,10-ethyleneisoalloxazinium chloride (2a).** mp 191–195°C; λ_{max} (CH_3CN) 340, 388 nm; ^{1}H NMR (CF_3COOH/CD_3COOD) δ 4.95 (t, 2H, J=8.9 Hz), 5.57 (t, 2H, J=8.9Hz), 8.20 (d, 1H, J=8.8 Hz), 8.47 (d, 1H, J=8.8 Hz), 8.79 (s, 1H); ^{13}C NMR (CF_3COOH-CD_3COOH, 6:1) δ 46.3, 52.2, 119.1, 131.4, 136.5, 122.8 (q, J[^{19}F, ^{13}C]=272.3 Hz, CF_3), 131.8, 134.3 (*C-4a), 135.1 (q, J[^{19}F,^{13}C]=35.5 Hz, C-7), 141.1, 145.3, 146.4, 158.8. **[4,10a-$^{13}C_2$]7-(Trifluoromethyl)-1,10-ethyleneisoalloxazinium chloride (2b).** mp 192–195°C; λ_{max} (CH_3CN) 340, 388 nm; ^{1}H NMR (CF_3COOH/CD_3COOD) δ 4.96 (t, 2H, J=8.7 Hz), 5.59 (t, 2H, J=8.7 Hz), 8.21 (d, 1H, J=8.8 Hz), 8.48 (d, 1H, J=8.8 Hz), 8.80 (s, 1H); ^{13}C NMR (CF_3COOH-CD_3COOD, 6:1) 46.3, 52.2, 119.1, 131.4, 136.4, 122.8 (q, J[^{19}F, ^{13}C]=271.6 Hz, CF_3), 131.8, 134.2, 135.0 (q, J[^{19}F,^{13}C]=35.7 Hz, C-7), 141.1, 145.3 (d, J=10.1 Hz, *C-10a), 146.4, 158.8 (d, J=10.1 Hz, *C-4).

4.5. Reaction of the ^{13}C-labeled 7-(trifluoromethyl)-flavinium chlorides 2a and 2b with benzylamine (or ^{15}N-labeled benzylamine): detection of C^{10a} adducts and subsequent reaction evolution

(i) ^{1}H NMR condition: The unlabeled (**2**) or [4a-^{13}C]-labeled flavinium chloride **2a** (4.1 mg, 0.012 mmol) and benzylamine (2.5 mg, 0.024 mmol) in acetonitrile-d_3 (0.6 mL) were mixed in a NMR tube at 25°C and the spectrum was recorded after 3 min: ^{1}H NMR (CD_3CN) δ 2.96 (br t, 1H), 3.38 (dd, 1H, J=13.8, 5.4 Hz, C-10a benzylic), 3.43 (dd, 1H, J=13.8, 5.4 Hz, C-10a benzylic), 3.75–3.95 (3H), 4.02–4.11 (m,1H), 6.86 (d, 1H, J=8.7 Hz), 7.05–7.08 and 7.15–7.20 (5H), 7.59 (dd, 1H, J=8.7, 1.9 Hz), 7.91 (d, 1H, J=1.9 Hz). (ii) ^{13}C NMR condition: The [4,10a-$^{13}C_2$]- or [4a-^{13}C]-labeled flavinium chloride **2b** or **2a** (40.6 mg, 0.12 mmol) and benzylamine or ^{15}N-benzylamine (25.7 mg, 0.24 mmol) were quickly mixed in acetonitrile (6 mL) at 25°C. The mixture was filtered to remove benzylamine hydrochloride and concentrated in vacuo. Without delay, the oily residue was dissolved in DMSO-d_6 and analyzed by ^{13}C NMR, revealing generation of the C^{10a} adducts: **[4a-^{13}C]7-CF_3-Fl$^+$Cl$^-$/benzylamine (7a)**: ^{13}C NMR (DMSO-d_6) δ 41.2, 44.3, 44.7, 78.5, 113.8, 118.4 (q, J[^{19}F, ^{13}C]=32.5 Hz, C-7), 124.5 (q, J[^{19}F,^{13}C]=270.6 Hz, CF_3), 126.5, 126.7, 127.4, 127.7, 128.4, 130.2, 136.9, 139.1 (*C-4a), 139.5, 148.6, 161.8. **[4a-^{13}C]7-CF_3-Fl$^+$Cl$^-$/^{15}N-benzylamine (7c)**: ^{13}C NMR (DMSO-d_6) δ 41.2, 44.3 (d, J=4.3 Hz, *NCH_2Ph), 44.7, 78.5 (d, J=6.6 Hz, C-10a), 113.8, 118.4 (q, J[^{19}F,^{13}C]=32.5 Hz, C-7), 124.5 (q, J[^{19}F, ^{13}C]=270.6 Hz, CF_3), 126.5, 126.7, 127.4, 127.7, 128.4, 130.2, 136.9, 139.1 (d, J=4.5 Hz, *C-4a), 139.5, 148.6, 161.8. **[4,10a-$^{13}C_2$]7-CF_3-Fl$^+$Cl$^-$/benzylamine (7b)**: ^{13}C NMR (DMSO-d_6) δ 41.2, 44.3, 44.7, 78.5 (d, J=8.6 Hz, *C-10a), 113.8, 118.4 (q, J[^{19}F,^{13}C]=32.5 Hz, C-7), 124.5 (q, J[^{19}F, ^{13}C]=270.6 Hz, CF_3), 126.5, 126.7, 127.5, 127.7, 128.4, 130.3, 136.9, 139.2, 139.5, 148.6, 161.8 (d, J=8.6 Hz, *C-4). **[4,10a-$^{13}C_2$]7-CF_3-Fl$^+$Cl$^-$/^{15}N-benzylamine (7d)**: ^{13}C NMR (DMSO-d_6) δ 41.2, 44.3 (d, J=4.6 Hz, *NCH_2Ph), 44.7, 78.5 (dd, J=8.6, 6.6 Hz, *C-10a), 113.8, 118.4 (q, J[^{19}F,^{13}C]=32.5 Hz, C-7), 124.5 (q, J[^{19}F,^{13}C]=270.6 Hz, CF_3), 126.5, 126.7, 127.5, 127.8, 128.4, 130.3, 136.9, 139.2 (d, J=4.1 Hz, C-4a), 139.5, 148.6, 161.8 (d, J=8.6 Hz, *C-4).

Following acquisition of the ^{13}C NMR spectra of the C^{10a} adducts **7**, the NMR tube reactions were allowed to proceed at 25°C in some cases, whereas in other cases the temperature of the NMR probe was raised to 70°C for 10 minutes to effect complete conversion of these initial adducts to a mixture of spirohydantoin amidine rearrangement products **8** (see Table 1 for the key NMR spectral data) or a mixture of the benzylamine-trapped dehydrogenation product ($PhCH{=}NCH_2Ph$), verified by its characteristic ^{1}H NMR absorptions at 4.76 δ (2h) and 8.43 δ (1H), and reduced dihydroflavin **12**, verified by isolation of its N^5-acetyl derivative[23] by quenching the reactions with Ac_2O under argon.

4.6. Isolation of 3′,4′-dihydro-3′-propylimino-3,4′-ethylene-7′-(trifluoromethyl)spiro-[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (9)

To a suspension of **2** (52 mg, 0.15 mmol) in acetonitrile (12 mL) was added a solution of *n*-propylamine (41 μL, 0.50 mmol) under an argon atmosphere. After the mixture was stirred at 50°C for 2 h, the solvent was removed under reduced pressure, and the remaining reddish oil was quenched by adding 6 mL CF_3COOH to give a yellow solution. The organic solvent (CF_3COOH) was evaporated under reduced pressure, and the oily residue was applied to a bed of AMBERLITE IRC-50 (weakly acidic carboxylic type) cation exchange resin. The neutral organic product was then washed off the column with $CHCl_3$ to afford 4.3 mg (7.8%) of **9**: λ_{max} (H_2O) 290 nm; ^{1}H NMR (CD_3OD) δ 0.85 (t, J=7.4 Hz, 3H), 1.42 (2H), 2.46 (m, 1H), 2.65 (m, 1H), 4.15–4.37 and 4.59–4.69 (4H), 7.44 (d, 1H, J=8.4 Hz), 7.70 (dd, 1H, J=8.4, 1.8 Hz), 8.10 (d, 1H, J=1.8Hz); ^{13}C NMR (CD_3CN) δ 11.6, 23.4, 44.1, 46.8, 48.2, 73.7 (C-2′), 117.6, 121.3, 124.5, 124.0, 124.6 (q, J[^{19}F,^{13}C]=271.6 Hz,CF_3), 128.0 (q, J[^{19}F,^{13}C]=33.5 Hz), 130.9, 153.9, 157.3, 168.4; FABMS *m/z* (relative intensity) 367 (M^+, 13), 310 (M^+-$NCH_2CH_2CH_3$, 100); HRMS (FAB) *m/z* calcd for $C_{16}H_{16}N_5O_2F_3$ 367.1258, found 367.1267.

4.7. Isolation of 3′,4′-dihydro-3′-diethyliminium-3,4′-ethylene-7′-trifluoromethylspiro-[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (10)

To a solution of **2** (244 mg, 0.71 mmol) in acetonitrile (36 mL) was added diethylamine (223 μL, 2.1 mmol) under an argon atmosphere. After the mixture was stirred at 50°C for 30 min, the solvent was removed under reduced pressure, and the remaining reddish oil was quenched by adding 12 mL CF_3COOH to give a yellow solution. The organic solvent (CF_3COOH) was evaporated under reduced pressure, and the oily residue was then recrystallized from isopropyl alcohol to give 166 mg (61%) of **10** as a pale-yellow powder. mp 158–160°C, dec.; λ_{max} (H_2O) 292 nm; ^{1}H NMR (CD_3OD) δ 0.99 (t, 6H, J=7.1 Hz), 2.79 (4H), 4.17–4.34 and 4.68–4.74 (4H), 7.42 (d, 1H, J=8.4 Hz), 7.70 (dd, 1H, J=8.4, 1.9 Hz), 8.02 (d, 1H, J=1.9 Hz); ^{13}C NMR (CD_3OD) δ 14.3, 42.5, 47.5, 48.0, 78.1 (C-2′), 117.5, 121.5, 124.8, 125.0 (q, J[^{19}F,^{13}C]=271.3 Hz, CF_3), 125.5, 128.8 (q, J[^{19}F,^{13}C]=33.1 Hz), 131.8, 154.5, 157.8, 168.6; FABMS *m/z* (relative intensity) 382 (M^+, 100), 310 (M^+-N-$(CH_2CH_3)_2$, 25), 266 (15), 239 (15); HRMS (FAB) *m/z* calcd for $C_{17}H_{19}N_5O_2F_3$ 382.1491, found 382.1497.

4.8. Molecular modeling

3′,4′-Dihydro-3′-benzylimino-3,4′-ethylene-7′-trifluoromethylspiro[imidazolidine-4,2′(1′H)-quinoxaline]-2,5-dione (**8**) was built using *Builder* in Insight II 950 (BIOSYM/MSI of San Diego). The model was refined by energy minimization using the program *Discover* with the CFF91 forcefield. Steepest descent minimization was first applied until the maximum derivative was less than 10 kcal/Å, and the conjugate gradient method was used subsequently until the maximum derivative was 1 kcal/Å. Finally, the Newton va09a method was used until the maximum derivative was less than 0.001 kcal/Å.

4.9. Detection of the C^{10a} hydroxy adduct 14

A mixture of **2b** (40.6 mg, 0.12 mmol) and water (4.4 mg, 0.24 mmol) in pyridine-d_5 (0.6 mL) was achieved quickly at 25°C, and the ^{1}H NMR spectrum was quickly recorded: ^{1}H NMR (pyridine-d_5) δ 3.97 (m, 1H), 4.19 (2H), 4.39 (m, 1H), 7.04 (d, 1H, J=8.7 Hz), 7.61 (d, 1H, J=8.7 Hz), 8.08 (s, 1H); ^{13}C NMR (pyridine-d_5) δ 41.6, 44.4, 85.8 (d, J=8.6 Hz, *C-10a), 114.1, 121.1 (q, $J[^{19}F,^{13}C]$=32.4 Hz, C-7), 125.3 (q, $J[^{19}F,^{13}C]$=270.5 Hz, CF_3), 127.8, 127.9, 132.1, 136.5, 142.2, 150.6, 162.5 (d, J=8.6 Hz, *C-4).

4.10. Reaction of 2 with amines in CD_3OD affords the C^{10a} methoxy adduct 16

To a CD_3OD (0.6 mL) solution containing **2** (100 mM) in an NMR tube at 25°C was added the same concentration of either benzylamine, propylamine, diethylamine, triethylamine, or NH_4OH (100 mM), and NMR spectra were immediately recorded. Regardless of amine identity the same initial spectrum was observed (omitting the amine signals): ^{1}H NMR (CD_3OD) δ 4.04 (3H), 4.42 (m, 1H), 7.28 (d, 1H, J=8.7 Hz), 7.75 (d, 1H, J=8.7 Hz), 7.95 (s, 1H); ^{13}C NMR (CD_3OD) δ 43.5, 47.6, 90.6 (C-10a), 115.9, 123.5 (q, $J[^{19}F,^{13}C]$=33.5 Hz, C-7), 127.8 (q, $J[^{19}F,^{13}C]$=270.5 Hz, CF_3), 128.5, 129.7, 132.6, 137.8, 139.9, 150.7, 163.1. The reactions were further monitored at various times over 24 h in order to observe the stable amine-derived products.

4.11. Isolation of methyl 7-(trifluoromethyl)-1,2-dihydroimidazo[1,2-a]quinoxaline-4-carboxylate (17)

By using the above procedure with a 1:1 molar ratio of benzylamine (6.4 mg, 0.06 mmol) and **2** (20.7 mg, 0.06 mmol) in CD_3OD (or CH_3OH), a stable spectrum was obtained after 24 h. The mixture was concentrated in vacuo, and $CHCl_3$ (10 mL) was added to the residue. The $CHCl_3$ layer was extracted with 2N HCl in saturated aqueous NaCl (10 mL). The spectrum of the $CHCl_3$ layer showed signals corresponding only to benzylurea. The water layer was then evaporated to afford a yellow powder containing **17** and benzylamine·HCl. The mixture displayed (omitting the signals for $PhCH_2NH_3^+Cl^-$): ^{1}H NMR (CD_3OD) δ 4.12 (s, 3H, when using CH_3OH rather than CD_3OD), 4.43 (t, 2H, J=10.0 Hz), 5.01 (t, 2H, J=10.0 Hz), 7.94 (d, 1H, J=8.5 Hz), 8.28 (d, 1H, J=8.5 Hz), 8.48 (s, 1H); ^{13}C NMR (CD_3OD) δ 46.1, 54.6, 118.3, 124.7 (q, $J[^{19}F,^{13}C]$=271.6 Hz), 130.1 (q, $J[^{19}F,^{13}C]$=33.0 Hz), 130.2, 133.3, 133.9, 135.7, 137.9, 149.9, 163.1. EIMS *m/z* (relative intensity) 300 (M^+, 100), 238 (81), 212 (52), 170 (8); HRMS (EI) *m/z* calcd for $C_{13}H_7N_3O_2D_3F_3$ 300.0914, found 300.0918.

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