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Executing and Rationalizing the Synthesis of a Difluorinated Analogue of a Ring-Expanded Calystegine B_2

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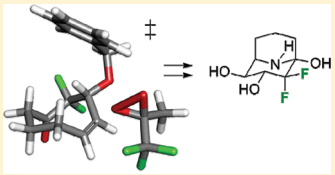
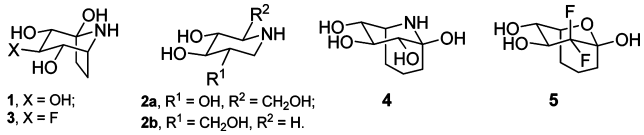
Supporting Information

ABSTRACT: A difluorinated analogue of a ring-expanded calystegine B_2 and some *N*-protected species were prepared via microwave-mediated transannular ring-opening of an epoxyketone. The diastereofacial selectivity of the epoxidation reaction, which delivers the key intermediate, and the regioselectivity of the transannular reactions were analyzed by density functional theory (DFT) methods. The epoxidation stereoselectivity arises from simple steric control, whereas the ring-closure reactions are subject to thermodynamic control.



INTRODUCTION

Calystegine B_2 **1** is a naturally occurring nortropane-type iminosugar related to 1-deoxynojirimicin **2a** and isofagomine **2b**. The calystegines have attracted attention from groups interested in their synthesis[1,2] and from others who seek to develop and understand the modes of action of glycosidase inhibitors.



1, X = OH; **3**, X = F

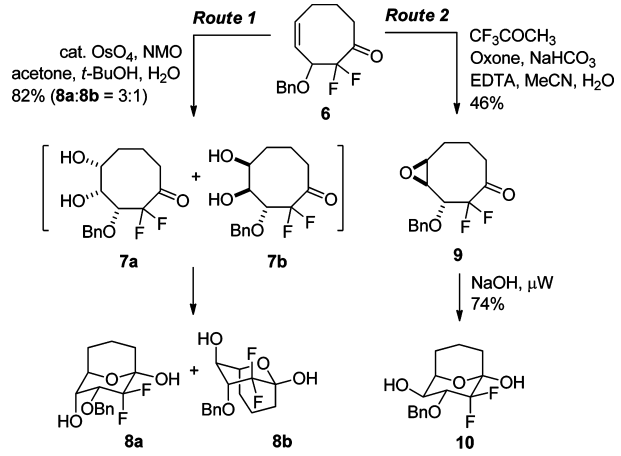
2a, R^1 = OH, R^2 = CH_2OH; **2b**, R^1 = CH_2OH, R^2 = H.

4

5

Iminosugars[3] are usually protonated at physiological pH and thus resemble the oxacarbenium ion intermediates (or oxacarbenium-ion-like transition states) involved in cleavage of glycosidic bonds; the importance of glycosidase action in a range of diseases (including cancer and metabolic diseases) has ensured a high level of interest in glycosidase inhibitors.[4−7] Structurally modified calystegines have attracted some attention,[8,9] including a fluorinated analogue **3**[10] and ring-expanded analogue **4** from the Leiden group.[11] Although **4** was not a glycosidase inhibitor, it is a very attractive multifunctional scaffold, presenting a range of different functional groups for derivatization. We prepared closely related bicyclic pentopyranose analogues including **5**;[12] alkene **6** was prepared via ring-closing metathesis, and alkene dihydroxylation afforded **7** (a mixture of diastereoisomers), which collapsed to bicyclic hemiacetals **8a** and **8b** (Scheme 1, route 1).[13] Scaffolds of this type are of interest because of the locked conformation and the high level of functionality; the presence of the CF_2 center also adds value by labeling the molecule for detection by ^{19}F NMR spectroscopy. Transannular reaction of one of the hydroxyl groups in **7** with the ketone secured the [3.3.1] bicyclic framework in **8**, a strategy also exploited by the Leiden group and explored by Paquette and Zhang.[14]

Scheme 1. Preparation of Protected Pentopyranose Analogues via Transannular Reactions



We also used the alternative strategy of transannular opening of epoxide **9** to prepare related compounds.[15] Reversible nucleophilic addition to the ketone carbonyl group followed by attack of a hemiacetal or hydrate oxygen at epoxide carbon transformed **9** to **10** (Scheme 1, route 2).

In this manuscript, we demonstrate the formation of a difluorinated analogue of a ring-expanded calystegine B_2 and describe our investigations of selected mechanistic aspects of the epoxidation and transannular chemistry more fully.

RESULTS AND DISCUSSION

Epoxide **9** was prepared following our published procedure; a single (racemic) *trans*-product was obtained from the dioxirane oxidation of alkene **6**. In the published syntheses, epoxides were reacted with aqueous ammonia in the microwave. Following workup, crystals were grown from the crude products, and the

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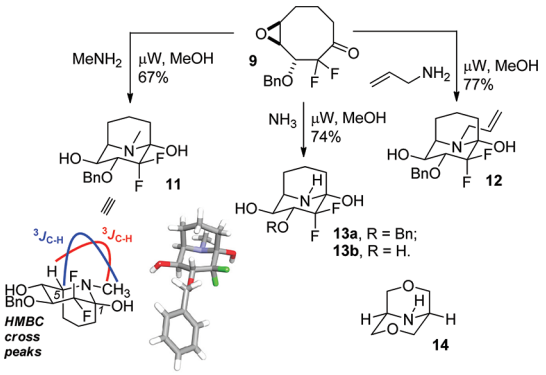
crystal structures were solved. Oxygen and nitrogen atoms can be difficult to distinguish unambiguously from diffraction data, and incorrect assignment was a distinct possibility. Microwave reaction of **9** with methylamine in methanol occurred smoothly; the ^{19}F NMR spectrum of the crude material revealed that a single product had been formed. The product afforded crystals of sufficient quality for analysis by X-ray crystallography, and the product was shown unambiguously to be the bridged bicyclic amine **11** (Scheme 2).

Scheme 2. Transannular Epoxide Opening with Ammonia and Primary Amines



HMBC analysis proved an effective way of demonstrating the regioselectivity of the reaction, with the observation of a strong cross peak between *N*-CH_3 and C-5. Methanolic allylamine effected the reaction, again affording the *N*-bridged bicyclic product **12** (confirmed by HMBC). Carrying out the reaction in methanolic ammonia also afforded the *N*-bridged secondary amine product **13a**. HMBC cannot be used in this case, but the H-5 proton can be assigned unambiguously in **13a**, and upon addition of *d*-TFA to an NMR sample, the chemical shift of the H-5 triplet (3.07 ppm) moved to 3.75 ppm. Similar chemical shift changes occurred upon TFA treatment of a solution of **11**, as the center of the H-5 multiplet (3.15−3.22 ppm) moved from 3.18 ppm to 3.70 ppm, consistent with protonation at nitrogen in both cases and indicative of the same connectivity. Park reported a 0.5 ppm shift change of the bridgehead methine proton signal of related amine **14** upon protonation at nitrogen.[16]

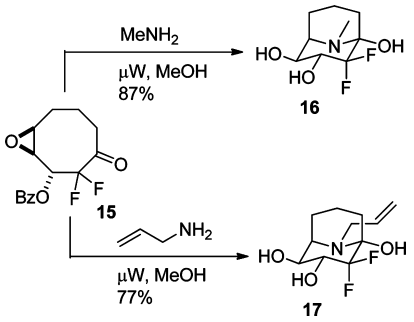
Unfortunately, epoxide ring-opening failed with enantiomerically enriched *α*-methylbenzylamine; we had hoped to achieve resolution by separating diastereoisomeric secondary amines. Epoxide **9** was consumed under the usual conditions, but the products were of very limited solubility and proved intractable.

Benzoate **15** can also be used successfully in this chemistry if a deprotected product is sought; aminolysis of the ester occurs during the ring-opening reaction allowing **16** (87%) and **17** (77%) to be isolated directly (Scheme 3).

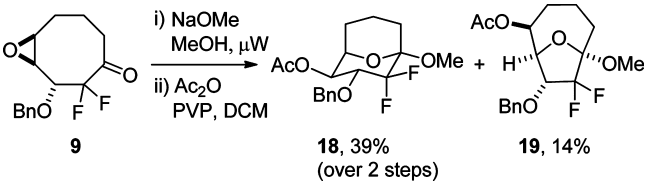
Unfortunately, reacting **15** with ammonia formed a mixture of product and side products from which we were unable to obtain clean material by chromatography.

In all cases of epoxide ring-opening with amines, [3.3.1] products were the only ones observed. As shown in Scheme 4, when **9** was instead reacted with sodium methoxide, a mixture of products was isolated; [3.3.1]-product **18** was the major product, but a significant quantity of [4.2.1] product **19** was also isolated. On the basis of related systems, formation of **18** was expected to be much more favorable. The mechanism of

Scheme 3. Epoxybenzoate Transannular Ring-Opening



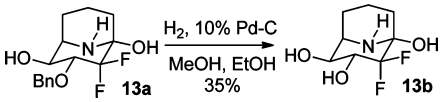
Scheme 4. Formation of a Mixture of Ring-Opening Regioisomers



formation of **18** and **19** can only involve methoxide attack at the ketone carbonyl group and subsequent transannular attack of the hemiacetal conjugate base on the epoxide.[12] This pathway relieves the strain arising from the epoxide and the eight-membered ring in the same step and should therefore be strongly favorable.

Deprotection of **13a** was achieved via hydrogenolysis in high crude yield under known conditions (Scheme 5).[12]

Scheme 5. Final Deprotection to Form 13b



Although a single product was obtained (by ^{19}F NMR), the yield of pure **13b** was low, with material lost during the chromatography. This amine is more polar than **16** or **17**, which may account for this difference. This sequence delivers difluorinated analogue **13b** of a ring-expanded calystegine B_2 through a highly stereoselective epoxidation and a fully chemo- and regioselective ring-opening.

Two selectivity issues arise from this route: the first concerns the stereoselectivity of the dioxirane oxidation, and the second encompasses the chemo- and regioselectivity of epoxide ring-opening. As cyclooctenone **6** and epoxides **9** and **15** are rich in functionality and conformationally fluxional, we decided to carry out electronic structure calculations to begin to understand aspects of the reactions more fully.

Epoxidation. Dioxirane oxidations of alkenes are of considerable interest because they can deliver highly enantiomerically enriched epoxides.[17,18] They occur on relatively flat (or soft) energy surfaces, which has made the calculation of accurate energy barriers rather difficult; recent work by Friesner and Breslow[19] showed that although the B3LYP[20,21] functional gave an adequate description of dioxirane oxidations of alkenes in many cases,[22] the UB3LYP method generally provided a more accurate account of energetic differences between diastereoisomeric epoxidation

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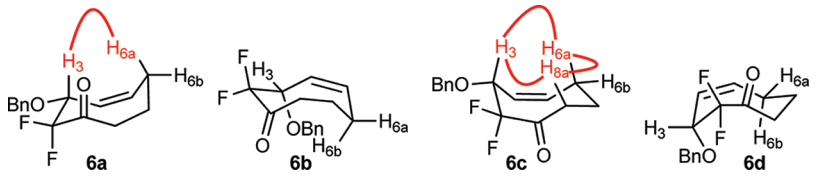

Figure 1. Boat−chair conformers identified for alkene **6**.

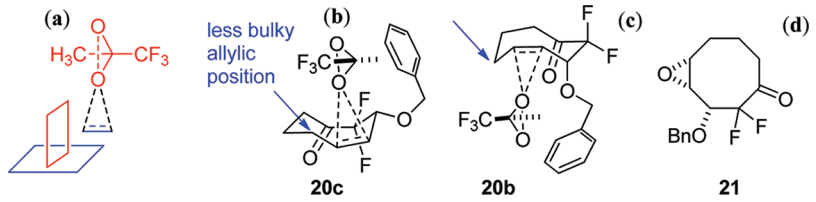

Figure 2. (a) *Spiro*-transition state arrangement; (b) productive mode of attack on the alkenyl group showing transition state **20c**; (c) higher energy transition state **20c**; and (d) *cis*-epoxide **21** (not observed) formed via transition state **20b**.

transition states. We have investigated the formation of **9** using electronic structure calculations, deploying the B3LYP functional and the UB3LYP method.

In a previous publication, we used a combination of Monte Carlo searching, electronic structure calculations, and low temperature ^{19}F, ^{1}H, and 2D (^{1}H−^{1}H) ROESY NMR to characterize the conformational space populated by related cyclooctenones.[23]

Alkene **6** is fluxional even at low temperature (the ^{19}F NMR spectrum is broad at 213 K), so J_{H-F} coupling constant analysis cannot be used to help distinguish between conformers. Four boat−chair conformers (**6a−d**), which were quite close in energy, were found by conformational searching using Spartan '08[24] (Figure 1). The NOESY spectrum provides clear experimental evidence for the population of **6c** with strong cross peaks between H-3, H-6$_a$, and H-8$_a$; the molecule also adopts this conformation in the crystal. Conformer **6a** could also be present but cannot be distinguished from **6c** on the basis of the NOESY spectrum. Benzyl ethers of each of the four boat−chair conformers were constructed, and the conformer distribution algorithm in Spartan'08 was used to scan the space available to the benzyl group (with the cyclooctane ring heavy atoms frozen). A set of 12 conformers was generated and optimized (B3LYP/6-31G*, gas phase) with nine conformers within 1 kcal mol^{-1} of the lowest energy species. We assumed that the *spiro*-transition state (Figure 2a) described by Houk[25,26] (and more recently by Werz[27] and Friesner and Breslow[19]) would be a good starting geometry for the epoxidation reaction and constructed a transition state template for 3-methyl-3-(trifluoromethyl)dioxirane oxidation of ethene (B3LYP/6-31G*). The template was incorporated into each of the 12 conformers; the methyl and trifluoromethyl groups were located on the unsubstituted and less hindered face of the alkene (Figure 2b), and the bulkier trifluoromethyl group was located closer to the smaller CH_2 center and away from the CHOBn methine (Figure 2c), consistent with Houk's model.

Single point energies were calculated for each conformer. From these, the lowest energy transition state **20c** leading to the *trans* major product **9** (from conformer **6c**), the lowest energy transition state **20b** leading to the *cis* (unobserved) product **21** (from conformer **6b**), and the matching starting materials and products were selected for optimization calculations using Gaussian 09 (B3LYP, UB3LYP, and M06 levels of theory with 6-31G* and 6-31+G* basis sets, gas phase, and PCM).[28] All ground and transition state structures in this study were verified by vibrational frequency analysis. Table 1 summarizes the results.

Table 1. Calculated Free Energies for the Epoxidation of 6 to the *trans* (9) and *cis* (21) Products via Transition State 20

method	G_{rel} (kcal mol^{-1})			
gas phase	**6c**[a]	**6b**	**20c**	**20b**
B3LYP/6-31G*	0.00	0.28	19.39	20.99
B3LYP/6-31+G*	0.00	0.91	20.95	22.02
UB3LYP/6-31G*	0.00	0.27	19.39	20.99
UB3LYP/6-31+G*	0.00	0.91	20.95	22.02
M06/6-31G*	0.00	0.47	16.75	17.45
M06/6-31+G*	0.00	1.96	17.02	19.08
PCM[b]				
B3LYP/6-31G*	0.00	1.42	19.32	19.85
B3LYP/6-31+G*	0.00	1.45	21.25	21.55
UB3LYP/6-31G*	0.00	1.35	19.25	19.78
UB3LYP/6-31+G*	0.00	1.44	21.25	21.55
M06/6-31G*	0.00	0.72	15.91	17.13
M06/6-31+G*	0.00	2.78	15.60	17.81

[a]Values normalized to the energy of **6c** in all cases. [b]The dielectric constant used was ε = 48 as described by Friesner and Breslow.[19]

The energies obtained with the B3LYP and UB3LYP levels of theory were very similar, and the energy differences for the pathways were consistently greater when the 6-31+G* basis set was employed. The treatment of solvation using the polarizable continuum model (PCM) method[29] discriminates consistently between conformers in favor of **6c** by ca. 1.4 kcal mol^{-1}; **6c** is observed in the NOESY spectrum. However, the normalized barrier heights on the two pathways lie within the usual error bars (±0.5 kcal mol^{-1}) for electronic structure calculations, so the facial selectivity would appear to arise solely from the energetic advantage enjoyed by boat−chair **6c**. Indeed, the barriers to oxidation of **6b** are lower at these levels of theory.

We noted that the methine proton in **6c** was relatively close (at 2.61 Å) to the quaternary aryl carbon and wondered if the well-documented inability of the B3LYP functional to account for dispersive interactions[30] might result in an underestimation of the stability of this conformer. Reoptimization of **6c** (and **20c**) using the pure meta-GGA functional M06 developed by Truhlar and co-workers[31,32] changed the geometry of **6c**,

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contracting the C−H···C(aryl) distance significantly to 2.35 Å and diverging the energies of **6b** and **6c** to 2.78 kcal mol^{-1} apart with the higher basis set. Although the barrier heights for the two diastereoisomers remain very similar, the M06/6-31+G* energy barriers are much (5 kcal mol^{-1}) lower at ca. 15 kcal mol^{-1} than those from the B3LYP or UB3LYP calculations (ca. 20 kcal mol^{-1}).

One feature of the geometry of the transition state structure is the very low degree of tilt away from the *spiro* geometry, indicating a highly synchronous mechanism. Usually, steric repulsions involving the bulkier dioxirane substituent cause tilt in the transition structure (for example, in Houk's early study); in this case, however, the measured ═CH···H—C and ═CH···F—C distances are 2.512 and 2.521 Å, respectively, at the B3LYP/6-31+G* level of theory (Figure 3). These distances shorten slightly to 2.437 and 2.480 Å at the M06/6-31+G* level of theory and in both cases reveal highly symmetrical transition states.

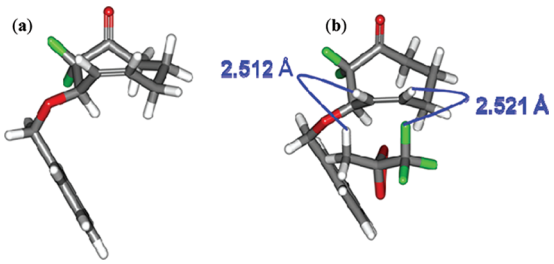


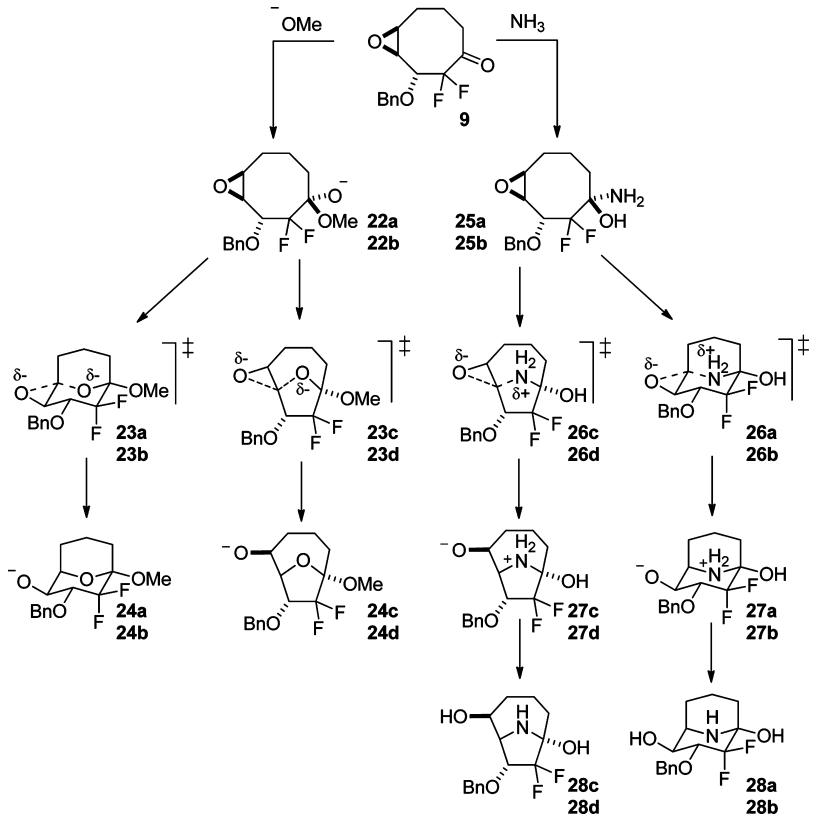
Figure 3. (a) The most favored cyclooctenone conformer leading to *trans*-product **6c** showing the open face of the alkene and (b) approach of the dioxirane in the transition state **20c**, highlighting ═CH···H—C and ═CH···F—C distances.

Whereas the former distance is greater than the sum of the van der Waals radii (2.40 Å for H and H, 2.714 Å for H and F), the latter is shorter than the sum; a favorable electrostatic interaction between the proton and fluorine atoms may allow the relatively close contact.[33]

The stereoselectivity observed in the dioxirane oxidation arises from the least hindered attack trajectory on the more open face of the most populated conformer and involves no special stereoelectronic effects. The synchronicity of the mechanism also explains the congruence between the energies calculated with the restricted and unrestricted B3LYP level of theory, and the radical character, which can only be described well by the UB3LYP method, is absent from these transition states. The M06 functional, which gives a better account of dispersive interactions, allows the transition structures to become more compact.

Ring-Closure Regiochemistry. Our previous publication assumed that the transannular epoxide ring-opening was irreversible and that control arose from the relative nucleophilicities of amino groups and oxyanions (with the latter more nucleophilic).[13] The stereoselective addition of small nucleophiles to the conformationally mobile ketone looks extremely unlikely, so the energetics of the various bicyclic closures must be considered. To gain some insight, we carried out electronic structure calculations of the reaction pathways involving both the oxyanion and amino group nucleophiles reacting with the epoxide **9**. The following strategy was

Scheme 6. Structures on the Ring-Opening Pathways with Oxyanion and Amino Group Nucleophiles[a]



[a]Hemiaminals **25a** and **25b** are ammonia adducts after proton loss.

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Table 2. Calculated Free Energies (B3LYP/6-31+G*/PCM (MeOH)) for the Transannular Reactions

oxyanion nucleophile				amine nucleophile			
			[3.3.1]-closure, G_{rel} (kcal mol^{-1})				
22a	(0.00)	**22b**	3.11	**25a**	(0.00)	**25b**	2.82
23a	16.02	**23b**	15.82	**26a**	23.57	**26b**	22.33
24a	−14.08	**24b**	−15.40	**27a**	6.92	**27b**	4.02
				28a	−19.04	**28b**	−22.57
			[4.2.1]-closure, G_{rel} (kcal mol^{-1})				
23c	15.06	**23d**	17.13	**26c**	24.89	**26d**	26.33
24c	−6.66	**24d**	−5.51	**27c**	16.14	**27d**	15.53
				28c	−16.68	**28d**	−16.82

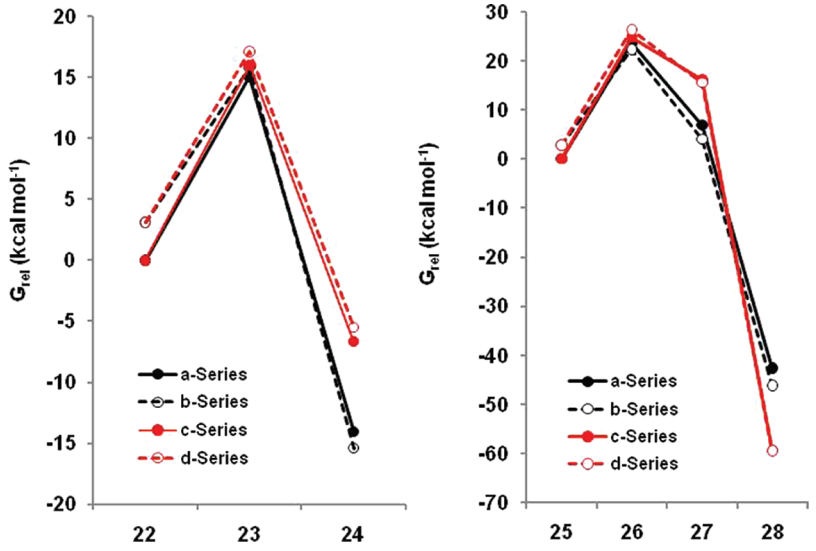

Figure 4. Free energy profiles for (a) oxyanion cyclization and (b) amino group cyclization.

followed: products of epoxide ring-opening were constructed and used as starting points for conformer distribution searches using the MMFF94 forcefield[34] and a Monte Carlo method. The lowest energy conformer for the ground state starting material and all other conformers within 4 kcal mol^{-1} were optimized (B3LYP/6-31G*, gas phase), and then a further selection was made. The bicyclic product structures arising from reaction of **9** with either the oxyanion or amine nucleophile were relatively rigid, and in all cases, only a second ring conformer was found within 2 kcal mol^{-1}.[35] Eight structures (**24a**−**d** and **27a**−**d**) were minimized in the first instance to allow comparison of the relative energies within the pathways (Scheme 6).

Transition structures for the transannular reactions (**23** and **26**) were optimized initially using a semiempirical method (RM1)[36] implemented in Spartan'08, and then distinct structures were reoptimized using Gaussian 09 (B3LYP/6-31+G*, PCM(methanol)). The transition structures were then collapsed during optimization to give matching precursor structures **22a**, **22b**, **25a**, and **25b**. The oxyanion cyclizations to **24a**−**d** were exergonic, whereas the amine ring closures to **27a**−**d** were endergonic to the zwitterions, even when the solvation model was applied (Table 2). We therefore ended our calculations with the neutral products (**28a**−**d**) following proton transfer so that the processes were exergonic overall. Structure **28a** appears earlier as **13a**, but we have renumbered in the interest of simplicity.

The results revealed some unexpected features of the cyclization. For the oxyanion cyclization, while the [3.3.1] products (**24a**, **24b**) were significantly (ca. 10 kcal mol^{-1}) more stable than the [4.2.1] regioisomers (**24c**, **24d**), as expected (Table 2), the energy barriers to [4.2.1] regioisomer formation were very similar to those for [3.3.1] closure (**23a**,**b** vs **23c**,**d**). However, there was quite a low ($\Delta G^{\ddagger}$ ca. 23 kcal mol^{-1}) barrier to ring-opening of the most easily formed [4.2.1] regioisomer **24c** (the reverse reaction), which suggests that the transannular cyclization that leads to **19** may be reversible. The presence of **19** must arise from incomplete thermodynamic control, given the large (8−10 kcal mol^{-1}) energy differences between [4.2.1] and [3.3.1] products (Figure 4).

When the nucleophile is an amino group, [3.3.1] product formation has a small kinetic advantage and leads to the lower energy zwitterions. The neutral [4.2.1]-bicyclic hemiaminals (**28c**,**d**) are only 2−5 kcal mol^{-1} more stable than the [3.3.1]-regioisomer products (**28a**,**b**), but [3.3.1] zwitterions **27a** and **27b** are 10 kcal mol^{-1} more stable than **27c** and **27d** and will therefore be present in much higher concentrations in the cyclization equilibrium (Figure 4). Protonation renders the reactions irreversible, so the partitioning of **25a** and **25b** in favor of the more stable zwitterions controls these reactions.

In basic media, competition between amine and oxyanion nucleophiles must be considered. Selective cyclization via the amino group can be understood by considering solvent effects on pK_a; when cyclization occurs to form **13**, hemiaminal **25** can present either an amino group or an hydroxyl group (or its conjugate base) as a nucleophile. The nucleophilicity hierarchy alkoxide > amine > alcohol would be anticipated. The amino group nucleophilicity (the pK_a of **29** was determined to be 5.87

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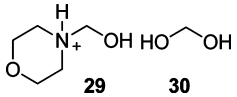
by titration[37]) will only be reduced further by the presence of the CF_2 center, so the amino group is likely to be fully neutral under the reaction conditions.



29 30

The pK_a of formaldehyde hydrate **30** is 12.78; a slightly higher value can be anticipated because nitrogen is less electronegative than oxygen, but the presence of the CF_2 center will lower the pK_a by up to 2.6 units,[38] so the O−H pK_a of **25** is estimated at 12.5. However, the solvent effect on pK_a has been neglected; transfer from water to alcohol solvent has a small effect on amine pK_a, but a much bigger one on oxygen acid ionization. Oxyanion stabilization is much less effective in alcohols, and oxygen acid pK_a-values are typically 5 units higher than in water.[39,40] It therefore seems likely that the competing nucleophiles are neutral hemiaminal nitrogen and hydroxyl groups and attack through nitrogen, and the formation of *N*-bridged species is therefore to be expected.

CONCLUSION

We have prepared a difluorinated analogue of a ring-expanded calystegine B_2 and a number of *N*-protected species via a microwave-mediated transannular ring-opening of an epoxyketone. The diastereofacial selectivity of the epoxidation reaction, which delivers the key intermediates, was very delicately balanced. One feature of the epoxidation was the close to perfect *spiro*-geometry, favored by the close approach of a fluorine atom in the dioxirane and a proton on the substrate. The transannular reactions appear to be under thermodynamic control; in the case of the oxyanions, the presence of [3.3.1] and [4.2.1]-products indicates that equilibrium was not established. For the hemiaminals, control was complete with the relative energies of the zwitterions decisive in determining the outcome.

EXPERIMENTAL SECTION

Epoxides **9** and **15** were prepared according to published procedures.[12]

Preparation of 3*R-Benzyloxy-2,2-difluoro-9-methyl-9-aza-1*S**,5*R**-bicyclo[3.3.1]nona-1*S**,4*S**-diol 11.** Epoxide **9** (0.2 mmol, 55 mg) was taken up in a solution of methylamine in methanol (2 mL of a 2 M solution) and sealed in a microwave vial (10 mL) containing a stirrer bead. The stirred solution was irradiated in the cavity of a microwave at 30 W power to hold a temperature of 100 °C for 30 min with a 10 min ramp up to temperature. After cooling, the tube was opened and the solution was concentrated under reduced pressure to a sticky yellow solid, which was purified by flash chromatography (silica, 70% ethyl acetate in hexane) to afford amine **11** as a colorless solid (41 mg, 67%): R_f (50% ethyl acetate/hexane) 0.31; mp 182−184 °C; ν_{max}(solid)/cm^{-1} 3401 br, 2956 s, 753 s, 697 s; ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.46−7.27 (m, 5 H), 4.91 (d, ^2J = 11.3 Hz, 1 H), 4.80 (d, ^2J = 11.3 Hz, 1 H), 4.03 (dt, J_{H-F} = 17.5, 9.2 Hz, J = 9.4 Hz, 1 H), 3.96 (m, J = 9.4, 5.8 Hz, 1 H), 3.07 (t, J = 4.8 Hz, 1 H), 1.85−1.55 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 138.2, 128.8 (s, 1 C), 128.7 (s, 1 C), 127.4 (s, 1 C), 119.9 (dd, $^1J_{C-F}$ = 253.2, 251.7 Hz, 1 C), 84.0 (dd, $^2J_{C-F}$ = 25.6, 19.2 Hz, 1 C), 91.4 (t, $^2J_{C-F}$ = 19.4 Hz, 1 C), 74.2 (d, $^4J_{C-F}$ = 2.4 Hz, 1 C), 71.3 (d, J_{C-F} = 11.2 Hz, 1 C), 60.0 (s, 1 C), 32.4 (d, $^4J_{C-F}$ = 2.4 Hz, 1 C), 22.6 (s, 1 C), 19.2 (s, 1 C), 13.3 (s, 1 C); ^{19}F NMR (282 MHz, CD_3OD) δ ppm −114.7 (dd, $^2J_{F-F}$ = 244.6 Hz, J_{H-F} = 9.2 Hz, 1 F), −124.3 (dd, $^2J_{F-F}$ = 244.6 Hz, J_{H-F} = 17.51 Hz, 1 F); HRMS calcd for $C_{16}H_{21}F_2NO_3$ + H$^+$, 314.1562; found (ESI$^+$, [M + H]$^+$), 314.1563; *m*/*z* (ESI$^+$) 314 (100%, [M + H]$^+$). Calcd for $C_{16}H_{21}F_2NO_3$: C, 61.3; H, 6.8; N, 4.5. Found: C, 61.4; H, 6.7; N, 4.4%.

Crystal data: $C_{16}H_{21}F_2NO_3$, crystal size 0.20 × 0.14 × 0.07 mm^3, M = 313.34, monoclinic, a = 6.7020(18) Å, b = 16.936(4) Å, c = 13.275(4) Å, α = 90°, β = 90.508(6)°, γ = 90°, U = 1506.7(7) Å^3, T = 150(2) K, space group $P2(1)/c$, Z = 4, μ(Mo Kα) = 0.111 mm^{-1}, 10673 reflections measured, 2644 [R(int) = 0.1284], which were used in all calculations. Final R indices [$F^2 > \sigma(F^2)$] $R1$ = 0.0584, $wR2$ = 0.0899; R indices (all data) $R1$ = 0.1157, $wR2$ = 0.1049.

Preparation of 3*R-Benzyloxy-2,2-difluoro-9-allyl-9-aza-1*S**,5*R**-bicyclo[3.3.1]nona-1*S**,4*S**-diol 12.** Compound was prepared from epoxide **9** (0.2 mmol, 56 mg) and allylamine (2 mL of a 2 M solution in methanol) as for **11**. The solution was concentrated under reduced pressure to a sticky yellow solid, which was purified by flash chromatography (silica, 70% ethyl acetate/hexane) to afford amine **12** as a colorless solid (52 mg, 77%): R_f (70% ethyl acetate/hexane) 0.32; mp 111−113 °C; ν_{max}(solid)/cm^{-1} 3503 br, 2943 s, 852 s, 746 s; ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.47−7.27 (m, 5 H), 5.79 (dddd, J = 17.2, 10.1, 7.6, 4.6 Hz, 1 H), 5.27−5.20 (m containing app. d, J = 17.2 Hz, 1 H), 5.14−5.08 (m containing app. d, J = 10.1 Hz, 1 H), 4.92 (d, ^2J = 11.3 Hz, 1 H), 4.80 (d, ^2J = 11.3 Hz, 1 H), 4.05 (dt, J_{H-F} = 17.4 Hz, J = 9.5 Hz, 1 H), 3.89 (dd, J = 9.5, 6.0 Hz, 1 H), 3.63−3.57 (m, 1 H), 3.30 (dd, ^2J = 14.3, J = 7.6 Hz, 1 H), 3.21−3.16 (m, 1 H), 1.81−1.60 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 138.2 (s, 1 C), 137.0 (s, 1 C), 127.9 (s, 1 C), 127.8 (s, 1 C), 127.4 (s, 1 C), 120.1 (dd, $^1J_{C-F}$ = 154.4, 152.0 Hz, 1 C), 115.4 (s, 1 C), 84.0 (dd, $^2J_{C-F}$ = 25.6, 19.2 Hz, 1 C), 81.6 (t, $^2J_{C-F}$ = 20.0 Hz, 1 C), 74.3 (d, $^4J_{C-F}$ = 2.4 Hz, 1 C), 71.5 (d, J_{C-F} = 9.6 Hz, 1 C), 54.5 (s, 1 C), 46.0 (d, $^4J_{C-F}$ = 1.6 Hz, 1 C), 24.7 (s, 1 C), 19.3 (s, 1 C), 13.6 (s, 1 C); ^{19}F NMR (282 MHz, CD_3OD) δ ppm −113.4 (dd, $^2J_{F-F}$ = 241.7 Hz, J_{H-F} = 8.21 Hz, 1 F), −124.7 (dd, $^2J_{F-F}$ = 241.7 Hz, J_{H-F} = 17.4 Hz, 1 F); HRMS calcd for $C_{18}H_{23}F_2NO_3$ + H$^+$, 340.1719; found (ESI$^+$, [M + H]$^+$) 340.1716; *m*/*z* (ESI$^+$) 340 (100%, [M + H]$^+$), 214 (15), 106 (29). Calcd for $C_{18}H_{23}F_2NO_3$: C, 63.7; H, 6.8; N, 4.1. Found: C, 63.6; H, 6.8; N, 4.1%.

Preparation of Benzyloxy-2,2-difluoro-9-aza-1*S,5*R**-bicyclo[3.3.1]nona-1*S**,4*S**-diol 13a.** Compound was prepared from epoxide **9** (0.44 mmol, 129 mg) and ammonia (5 mL of a saturated solution in methanol) as for **11**. The solution was concentrated under reduced pressure to a sticky yellow solid, which was purified by flash chromatography (100% ethyl acetate) to afford amine **13a** as a colorless solid (101 mg, 74%): mp 165−170 °C; R_f (100% ethyl acetate) 0.24. ν_{max}(solid)/cm^{-1} 3416 br, 2945 s, 1737 s, 1455 w, 1367 s, 1198 s, 1144 s, 1037 s, 905 s, 751 s, 695 s; ^{1}H NMR (300 MHz, CD_3OD) δ ppm 7.45−7.26 (m, 5 H), 4.91 (d, ^2J = 11.3 Hz, 1 H), 4.81 (d, ^2J = 11.3 Hz, 1 H), 4.02 (ddd, J_{H-F} = 18.0, 7.4, J 9.4, 1 H), 3.94−3.87 (m, 1 H), 3.22−3.15 (m, 1 H), 2.05−1.48 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 138.2 (s, 1 C), 127.9 (s, 1 C), 127.8 (s, 1 C), 127.3 (s, 1 C), 120.4 (dd, $^1J_{C-F}$ = 159.6, 149.3 Hz, 1 C), 81.1 (t, $^2J_{C-F}$ = 19.2 Hz, 1 C), 80.8 (d, $^2J_{C-F}$ = 20.0 Hz, 1 C), 74.5 (d, $^4J_{C-F}$ = 2.4 Hz, 1 C), 73.5 (d, J_{C-F} = 7.2 Hz, 1 C), 52.8 (s, 1 C), 30.3 (d, J_{C-F} = 2.4 Hz), 20.6 (s, 1 C), 19.0 (s, 1 C); ^{19}F NMR δ ppm (282 MHz, CD_3OD) −117.1 (1 F, dd, $^2J_{F-F}$ = 245.5, J_{H-F} = 7.4 Hz), −128.1 (1 F, ddd, $^2J_{F-F}$ = 245.5, J_{H-F} = 18.0, $^4J_{H-F}$ = 3.8 Hz); HRMS calc. for $C_{15}H_{20}F_2NO_3$ 300.1406; found (ESI$^+$, [M + H]$^+$), 300.1409; *m*/*z* (ESI$^+$) 300 (50%, [M + H]$^+$), 297 (59), 242 (47), 163 (14), 91 (100), 72 (95). Calcd for $C_{15}H_{19}F_2NO_3$: C, 60.2; H, 6.4; N, 4.7. Found: C, 60.16; H, 6.3; N, 4.6%).

Crystal data: $C_{15}H_{19}F_2NO_3$, crystal 0.33 × 0.20 × 0.10 mm^3, M = 299.31, orthorhombic, a = 11.428(3) Å, b = 24.352(6) Å, c = 10.474(2) Å, α = 90°, β = 90°, γ = 90°, U = 2915.0(12) Å^3, T = 150(2) K, space group $Pccn$, Z = 8, μ(Mo Kα) = 0.111 mm^{-1}, 19696 reflections measured, 2576 [R(int) = 0.0528], which were used in all calculations. Final R indices [$F^2 > 2\sigma(F^2)$] $R1$ = 0.0498, $wR2$ = 0.1134; R indices (all data) $R1$ = 0.0658, $wR2$ = 0.1194.

Preparation of 2,2-Difluoro-9-methyl-9-aza-1*S,5*R**-bicyclo-[3.3.1]nona-1*S**,3*R**,4*S**-triol 16.** Compound was prepared from epoxide **15** (0.135 mmol, 40 mg) in methylamine (1.3 mL of a 2 M solution in methanol) as for **11**. The solution was concentrated in vacuo to give a sticky yellow solid, which was purified by flash chromatography (100% ethyl acetate) to afford triol **16** as a colorless solid (37 mg, 87%): R_f (50% ethyl acetate/hexane) 0.07; mp 95−97

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°C; ν_{max}(solid)/cm^{-1} 3375 br,w, 2964 w, 2508 w, 2321 br, 1444 w, 1341 w, 1128 s, 999 s, 850 m, 780 s; ^{1}H NMR (300 MHz, CD_3OD) δ ppm 3.95 (dt, J_{H-F} = 18.8 Hz, J = 9.7 Hz, 1 H), 3.74 (dd, J = 9.7 Hz, $^4J_{H-F}$ = 5.5 Hz, 1 H), 2.95 (broad t, J = 4.6 Hz, 1 H), 1.72−1.46 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 119.2 (dd, $^1J_{C-F}$ = 252.4, 249.3 Hz, 1 C), 83.8 (dd, $^2J_{C-F}$ = 25.6, 18.4 Hz, 1 C), 73.9 (dd, $^2J_{C-F}$ = 22.4, 20.0 Hz, 1 C), 72.1 (dd, J_{C-F} =10.4, 1.6 Hz, 1 C), 60.0, 32.4 (d, $^4J_{C-F}$ = 2.4 Hz, 1 C), 22.5 (d, J_{C-F} = 1.6 Hz, 1 C), 19.3 (1 C), 13.1 (1 C); ^{19}F NMR (282 MHz, CD_3OD) δ ppm −119.9 (dd, $^2J_{F-F}$ = 243.6 Hz, J_{H-F} = 10.0 Hz, 1 F), −126.8 (ddd, $^2J_{F-F}$ = 243.6 Hz, J_{H-F} = 18.8 Hz, $^4J_{H-F}$ = 5.5 Hz, 1 F); HRMS calcd for $C_9H_{15}F_2NO_3$ + H$^+$, 224.1093; found (ESI$^+$, [M + H]$^+$), 224.1095; m/z (ESI$^+$) 224 (1%, [M]$^+$), 223 (11), 206 (12), 142 (18), 112 (100), 73 (60). Satisfactory microanalysis could not be obtained for this compound.

Preparation of 2,2-Difluoro-9-allyl-9-aza-1*S,5*R**-bicyclo-[3.3.1]nona-1*S**,3*R**,4*S**-triol 17.** Compound was prepared from epoxide **15** (0.15 mmol, 44 mg) in allylamine (1.5 mL of a 2 M solution in methanol) as for **11**. The solution was concentrated in vacuo to give a sticky yellow solid, which was purified by flash chromatography (100% ethyl acetate) to afford to give triol **17** as a colorless solid (29 mg, 77%): R_f (50% ethyl acetate) 0.23; mp 119−121 °C; ν_{max}(solid)/cm^{-1} 3406 br,s, 2944 s, 1400 w, 1342 m, 1155 m, 1015 s, 932 s, 852 s, 783 m; ^{1}H NMR (400 MHz, CD_3OD) δ ppm 5.79 (dddd, J = 17.2, 10.2, 7.5, 4.7 Hz, 1 H), 5.12 (ddd, J = 17.2 Hz, ^4J = 1.9 Hz, ^2J = 1.1 Hz, 1 H), 4.98 (ddd, J = 10.2 Hz, ^4J = 1.9 Hz, ^2J = 1.1 Hz, 1 H), 3.93 (ddd, J_{H-F} = 18.6, 9.8 Hz, J = 9.7 Hz, 1 H), 3.78 (ddt, J = 9.7, 5.8 Hz, $^4J_{H-F}$ = 1.5 Hz, 1 H), 3.60 (ddt, ^2J = 14.5 Hz, J = 4.7 Hz, ^4J = 1.9 Hz, 1 H), 3.30 (dd, ^2J = 14.5 Hz, J = 7.5 Hz, 1 H), 3.21−3.16 (m, 1 H), 1.82−1.59 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 137.0 (1 C), 119.3 (dd, $^1J_{C-F}$ = 254.0, 250.1 Hz, 1 C), 115.4 (1 C), 83.8 (dd, $^2J_{C-F}$ = 26.4, 20.0 Hz, 1 C), 74.0 (dd, $^2J_{C-F}$ = 22.4, 20.0 Hz, 1 C), 72.3 (d, J_{C-F} = 8.8 Hz, 1 C), 54.5 (1 C), 46.0 (1 C), 24.6 (d, J_{C-F} = 2.4 Hz, 1 C), 19.3 (1 C), 13.4 (1 C); ^{19}F NMR (282 MHz, CD_3OD) δ ppm −119.6 (dd, $^2J_{F-F}$ = 243.1 Hz, J_{H-F} = 9.8 Hz, 1 F), −126.8 (ddd, $^2J_{F-F}$ = 243.1 Hz, J_{H-F} = 18.6 Hz, $^4J_{H-F}$ = 1.5 Hz, 1 F); HRMS calcd for $C_{11}H_{17}F_2NO_3$ + H$^+$, 250.1249; found (ESI$^+$, [M + H]$^+$), 250.1250; m/z (ESI$^+$) 250 (100%, [M + H]$^+$), 132 (15). Calcd for $C_{11}H_{17}F_2NO_3$: C, 53.0; H, 6.9; N, 5.6. Found: C, 53.0; H, 6.9; N, 5.6%.

Preparation of 2,2-Difluoro-9-aza-1*S,5*R**-bicyclo[3.3.1]-nona-1*S**,3*R**,4*S**-triol 13b.** A solution of **13a** (0.247 mmol, 74 mg) in methanol (1 mL) was added to a suspension of 10% palladium on activated carbon (24 mg) in ethanol (2.4 mL). The atmosphere was removed and replaced several times with hydrogen from a balloon. The solution was stirred at room temperature for 48 h, and then the hydrogen atmosphere was removed and replaced with air. The solution was filtered through Celite, concentrated under reduced pressure, and then purified by flash chromatography (100% ethyl acetate) to afford bicyclic amine **13b** as a colorless solid (18 mg, 35%): R_f (100% ethyl acetate) 0.19; mp 222−224 °C; ^{1}H NMR (400 MHz, CD_3OD) δ ppm 4.06 (ddd, J_{H-F} = 19.4, 8.5 Hz, J 9.6, 1 H), 3.82−3.77 (m, simplifying to dd, J = 9.6, 5.9 Hz with ^{19}F decoupling, 1 H), 3.22−3.17 (m, 1 H), 2.04−1.96 (m, 2 H), 1.83−1.75 (m, 1 H), 1.67−1.51 (m, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 119.7 (dd, $^1J_{C-F}$ = 258.8, 246.9 Hz, 1 C), 80.7 (dd, $^2J_{C-F}$ = 24.0, 20.0 Hz, 1 C), 74.2 (d, J_{C-F} = 8.0 Hz, 1 C), 73.5 (t, $^2J_{C-F}$ = 20.8 Hz, 1 C), 52.7 (1 C), 30.2 (d, J_{C-F} = 3.2 Hz, 1 C), 20.5 (1 C), 19.1 (1 C); ^{19}F NMR (282 MHz, CD_3OD) δ ppm −121.8 (dd, $^2J_{F-F}$ = 243.7 Hz, J_{H-F} = 8.5 Hz, 1 F,), −130.6 (ddd, $^2J_{F-F}$ = 243.7 Hz, J_{H-F} = 19.4 Hz, $^4J_{H-F}$ = 4.0 Hz, 1 F); HRMS calcd for $C_8H_{13}F_2NO_3$ + H$^+$, 210.0936; found (ESI$^+$, [M + H]$^+$), 210.0937; m/z (CI$^+$) 210 (100%, [M + H]$^+$), 292 (3), 172 (4), 156 (5), 115 (4), 98 (6), 52 (13). Satisfactory microanalysis could not be obtained for this compound.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General experimental procedures, computational methods, NMR spectra (^{1}H, ^{13}C, ^{19}F) for **11**, **12**, **13**, **16**, **17**, and **20**, and crystal data for **11** and **13a**. Optimized geometries (coordinates), free energies, and imaginary frequencies (where appropriate) for all structures employed in this study are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Moosophon, P.; Baird, M. C.; Kanokmedhakul, S.; Pyne, S. G. *Eur. J. Org. Chem.* **2010**, 3337.

(2) Monrad, R. N.; Pipper, C. B.; Madsen, R. *Eur. J. Org. Chem.* **2009**, 3387.

(3) Stocker, B. L.; Dangerfield, E. M.; Win-Mason, A. L.; Haslett, G. W.; Timmer, M. S. M. *Eur. J. Org. Chem.* **2010**, 1615.

(4) Kato, A. K. A.; Wang, L.; Ishii, K.; Seino, J.; Asano, N.; Suzuki, T. *J. Biochem.* **2011**, *149*, 415.

(5) Aguilar, M.; Gloster, T. M.; Garcia-Moreno, M. I.; Mellet, C. O.; Davies, G. J.; Llebaria, A.; Casas, J.; Egido-Gabas, M.; Fernandez, J. M. G. *ChemBioChem* **2008**, *9*, 2612.

(6) Garcia-Moreno, M. I.; Mellet, C. O.; Fernandez, J. M. G. *Eur. J. Org. Chem.* **2004**, 1803.

(7) Gloster, T. M.; Davies, G. J. *Org. Biomol. Chem.* **2010**, *8*, 305.

(8) Kaliappan, K. P.; Das, P.; Chavan, S. T.; Sabharwal, S. G. *J. Org. Chem.* **2009**, *74*, 6266.

(9) Beniazza, R.; Desvergnes, V.; Mehta, G.; Blanchard, N.; Robert, F.; Landais, Y. *J. Org. Chem.* **2011**, *76*, 791.

(10) Csuk, R.; Prell, E.; Reissmann, S.; Korb, C. *Z. Naturforsch., B: J. Chem. Sci.* **2010**, *65*, 445.

(11) van Hooft, P. A. V.; Litjens, R.; van der Marel, G. A.; van Boeckel, C. A. A.; van Boom, J. H. *Org. Lett.* **2001**, *3*, 731.

(12) Miles, J. A. L.; Mitchell, L.; Percy, J. M.; Singh, K.; Uneyama, E. *J. Org. Chem.* **2007**, *72*, 12.

(13) Fawcett, J.; Griffiths, G. A.; Percy, J. M.; Pintat, S.; Smith, C. A.; Spencer, N. S.; Uneyama, E. *Chem. Commun.* **2004**, 302.

(14) Paquette, L. A.; Zhang, Y. L. *J. Org. Chem.* **2006**, *71*, 4353.

(15) Fawcett, J.; Griffith, G. A.; Percy, J. M.; Uneyama, E. *Org. Lett.* **2004**, *6*, 1277.

(16) Park, C. M. *J. Org. Chem.* **2006**, *71*, 413.

(17) Shi, Y. *Acc. Chem. Res.* **2004**, *37*, 488.

(18) Yang, D. *Acc. Chem. Res.* **2004**, *37*, 497.

(19) Schneebeli, S. T.; Hall, M. L.; Breslow, R.; Friesner, R. *J. Am. Chem. Soc.* **2009**, *131*, 3965. This paper provides extensive references to earlier work.

(20) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648.

(21) Lee, C. T.; Yang, W. T.; Parr, R. G. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1988**, *37*, 785.

(22) Bach, R. D.; Glukhovtsev, M. N.; Gonzalez, C.; Marquez, M.; Estevez, C. M.; Baboul, A. G.; Schlegel, H. B. *J. Phys. Chem. A* **1997**, *101*, 6092.

(23) Griffith, G. A.; Percy, J. M.; Pintat, S.; Smith, C. A.; Spencer, N.; Uneyama, E. *Org. Biomol. Chem.* **2005**, *3*, 2701.

(24) *Spartan'08*; Wavefunction, Inc.: Irvine, CA, 2008.

(25) Houk, K. N.; Liu, J.; DeMello, N. C.; Condroski, K. R. *J. Am. Chem. Soc.* **1997**, *119*, 10147.
(26) Jenson, C.; Liu, J.; Houk, K. N.; Jorgensen, W. L. *J. Am. Chem. Soc.* **1997**, *119*, 12982.
(27) Dufert, A.; Werz, D. B. *J. Org. Chem.* **2008**, *73*, 5514.
(28) Frisch, M. J.et al. *Gaussian 09*, Revision A.1; Gaussian, Inc.: Wallingford, CT, 2009. See the Supporting Information for the full reference.
(29) Miertus, S.; Tomasi, J. *Chem. Phys.* **1982**, *65*, 239.
(30) Raju, R. K.; Ramraj, A.; Hillier, I. H.; Vincent, M. A.; Burton, N. A. *Phys. Chem. Chem. Phys.* **2009**, *11*, 3411.
(31) Zhao, Y.; Truhlar, D. G. *Org. Lett.* **2007**, *9*, 1967.
(32) Zhao, Y.; Truhlar, D. G. *Acc. Chem. Res.* **2008**, *41*, 157.
(33) Armstrong, A.; Washington, I.; Houk, K. N. *J. Am. Chem. Soc.* **2000**, *122*, 6297.
(34) Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490.
(35) Both conformers were investigated for [3.3.1]- (**a** and **b** suffixes) and [4.2.1]-products (**c** and **d** suffixes); the lowest energy conformers have **a** and **c** suffixes, so a **d** suffix (**23d**, **26d**) refers to the higher energy [4.2.1]-product.
(36) Rocha, G. B.; Freire, R. O.; Simas, A. M.; Stewart, J. J. P. *J. Comput. Chem.* **2006**, *27*, 1101.
(37) Taylor, P. D. *Talanta* **1995**, *42*, 243.
(38) Ethanol pK_a is 15.9, difluoroethanol pK_a is 13.3; see: Hondal, R. J.; Zhao, Z.; Kravchuk, A. V.; Liao, H.; Riddle, S. R.; Yue, X.; Bruzik, K. S.; Tsai, M. D. *Biochemistry* **1998**, *37*, 4568. For other reports of the modulation of basicity at nitrogen through fluorination, see: (b) Newhouse, B. J.; Hansen, J. D.; Grina, J.; Welch, M.; Topalov, G.; Littman, N.; Callejo, M.; Martinson, M.; Galbraith, S.; Laird, E. R.; Brandhuber, B. J.; Vigers, G.; Morales, T.; Woessner, R.; Randolph, N.; Lyssikatos, J.; Olivero, A. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3488. (c) Rowley, M.; Hallett, D. J.; Goodacre, S.; Moyes, C.; Crawforth, J.; Sparey, T. J.; Patel, S.; Marwood, R.; Thomas, S.; Hitzel, L.; O'Connor, D.; Szeto, N.; Castro, J. L.; Hutson, P. H.; MacLeod, A. M. *J. Med. Chem.* **2001**, *44*, 1603. (d) Bohm, H. J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Muller, K.; Obst-Sander, U.; Stahl, M. *ChemBioChem* **2004**, *5*, 637. (e) Muller, K.; Faeh, C.; Diederich, F. *Science* **2007**, *317*, 1881.
(39) Sarmini, K.; Kenndler, E. *J. Biochem. Biophys. Methods* **1999**, *38*, 123.
(40) Rived, F.; Canals, I.; Bosch, E.; Roses, M. *Anal. Chim. Acta* **2001**, *439*, 315.