Cross-Linking of Proteins by 3-(Trifluoromethyl)-2,5-hexanedione. Model Studies Implicate an Unexpected Amine-Dependent Defluorinative Substitution Pathway Competing with Pyrrole Formation†

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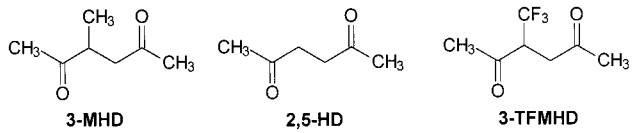
Protein modification by the neurotoxic γ-diketone 3-methyl-2,5-hexanedione (3-MHD) and its analogue 3-(trifluoromethyl)-2,5-hexanedione (3-TFMHD) was examined. Unlike 3-MHD, which forms lysine-based pyrroles that lead to autoxidation-dependent protein cross-linking, 3-TFMHD forms an autoxidatively inert pyrrole. The surprising finding that 3-TFMHD was nonetheless as effective as 3-MHD in cross-linking ribonuclease A suggested that protein lysine condensation with 3-TFMHD could take an alternate course competing with pyrrole formation. Model studies using neopentylamine led to the isolation of the expected 1-(2,2-dimethylpropyl)-2,5-dimethyl-3-(trifluoromethyl)pyrrole as well as the neopentylamine-3-TFMHD 2:1 adducts *N*,*N*′-bis(2,2-dimethylpropyl)-2-amino-3-acetyl-5-methylpyrrole (major) and *N*,*N*′-bis(2,2-dimethylpropyl)-3-(1-aminoethylidene)-5-methyl-4-pyrrolin-2-one (minor). The formation of these 2:1 adducts, the lysine analogues of which are believed to be mainly responsible for the observed protein cross-linking, is proposed to proceed via Schiff base formation, enamine fluoride elimination, second amine condensation, and hydrolysis.

Introduction

The neurotoxicity of *n*-hexane and methyl *n*-butyl ketone has traditionally been viewed as reflecting at least in part the condensation of their common γ-diketone metabolite, 2,5-hexanedione (2,5-HD), with lysine ε-amino residues of neurofilament (NF) proteins to form pyrroles via the Paal−Knorr condensation.[1−6] The resulting neurotoxic syndromes, which are associated with accumulation of aggregated NF at prenodal sites in large motor axons, also resemble naturally occurring neurological disorders. Although recent evidence suggests that axonal degeneration induced by 2,5-HD may be independent of NF accumulation,[7,8] pyrrole formation and possible subsequent cross-linking of other crucial neuronal proteins remains a primary consideration. Regarding the mechanism of NF accumulation, it remains unclear whether the key factor is simple pyrrole formation and the resulting physicochemical alteration of NF[4,5,9] or pyrrole autoxidation and the consequential covalent intermolecular cross-linking of NF.[10,11] Increasing the number of methyl groups on the γ-diketone is known to be associated both with an increased pyrrole formation rate and an increased tendency for autoxidation-dependent cross-linking.[12,13]



As part of an effort to elucidate the basis of NF aggregation, we synthesized various γ-diketones that would permit a dissociation between the rate of pyrrole formation and rate of pyrrole autoxidation.[14] One such γ-diketone was 3-(trifluoromethyl)-2,5-hexanedione (3-TFMHD), which was expected to form pyrroles at a rate comparable to that for the neurotoxic 3-methyl-2,5-hexanedione (3-MHD),[15] but the resulting trifluoromethyl-substituted pyrrole would be inert to autoxida-

† Abbreviations: EDC, 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide methiodide; 3-MHD, 3-methyl-2,5-hexanedione; 3-TFMHD, 3-(trifluoromethyl)-2,5-hexanedione; NAC, *N*-acetyl-L-cysteine; NF, neurofilaments; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; sulfo-NHS, *N*-hydroxysulfosuccinimide; TNBS; trinitrobenzenesulfonic acid.

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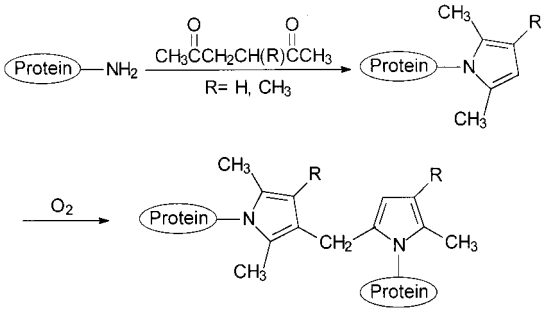
Scheme 1



tion. These expectations were borne out,[14] and 3-TFMHD was initially anticipated to be a useful probe for investigating the mechanism of γ-diketone-induced NF aggregation. However, an evaluation of the neurotoxicity of this compound in vivo was thwarted by its high systemic toxicity, and in vitro evaluation indicated that despite the inertness of 3-TFMHD-derived pyrroles, 3-TFMHD was as potent a protein cross-linking reagent as was 3-MHD. We disclose herein details of the reactions of proteins and amines with these two γ-diketones, including the elucidation of amine-3-TFMHD 2:1 adducts proposed to rationalize protein lysine-based cross-linking. On the basis of the structure of these adducts, a reasonable reaction mechanism for cross-linking is proposed involving an unexpected fluoride elimination initiated by imine−enamine tauomerization following amine condensation.

Results and Discussion

Cross-Linking of RNase A by 3-MHD and 3-TFMHD. The Effect of Added *N-Acetyl*-L-cysteine. Autoxidation of 2,5-dimethylpyrrole models or 2,5-dimethylpyrrolated protein leads to cross-linking reactions, the major mechanism of which involves the generation of pyrrole−pyrrole dimers containing a methylene bridge between C-2 and C-3 of the two pyrrole rings (Scheme 1, R = H).[16−19] Autoxidation in the presence of thiols, including *N*-acetyl-L-cysteine (NAC) and glutathione, leads to 3-mercaptopyrroles in competition with pyrrole−pyrrole coupling.[16,17,20] Inhibition of pyrrole−pyrrole autoxidative coupling by thiols also appears to result from hydrogen atom donation by the thiols.[17] Although the formation of 3-mercaptopyrroles suggests an alternative possibility for protein cross-linking involving trapping of lysine-derived pyrrole radicals by protein cysteine residues, this reaction has been observed only using low molecular weight thiols and has not been seen with protein-based cysteine.[17,19]

We previously reported that 3-MHD cross-links proteins, consistent with a mechanism involving formation of protein-bound 2,3,5-trimethylpyrroles and autoxidation of the latter to reactive intermediates capable of covalent coupling, as supported by the observed autoxidative dimerization of the independently generated primary-amine-derived 2,3,5-trimethylpyrroles.[14] Using RNase as a simple model for lysine-containing proteins of physiological relevance, 3-TFMHD was surprisingly also found to induce cross-linking even though the isolated primary-amine-derived 3-(trifluoromethyl)pyrroles were found to be resistant to oxidation.[14] To further investigate these two cross-linking processes, we evaluated the cross-linking of RNase in the absence and presence of NAC. Addition of NAC (20-fold excess over γ-diketone) resulted in complete inhibition of 3-MHD-induced RNase cross-linking but only partial inhibition of cross-linking by 3-TFMHD, as indicated by the level of RNase dimer formed (Figure 1).

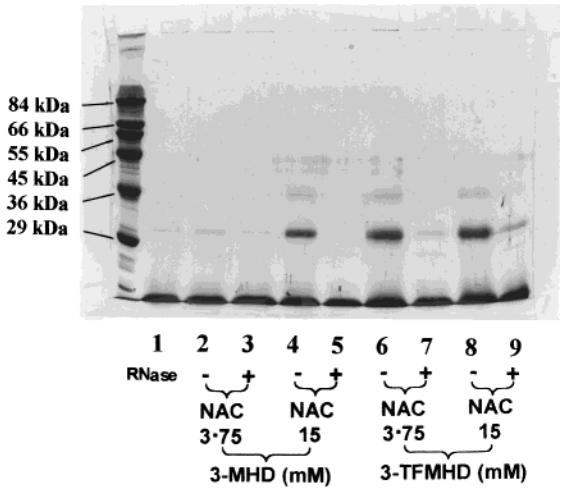


Figure 1. Inhibitory effects of NAC on 3-MHD and 3-TFMHD-induced RNase A cross-linking: control RNase A (lane 1), 3.75 (lane 2) and 15 mM (lane 4) 3-MHD-treated RNase A, 3.75 (lane 3) and 15 mM (lane 5) 3-MHD-treated RNase A in the presence of NAC (20-fold excess over 3-MHD), 3.75 (lane 6) and 15 mM (lane 8) 3-TFMHD-treated RNase A, and 3.75 (lane 7) and 15 mM (lane 9) 3-TFMHD-treated RNase A in the presence of NAC (20-fold excess over 3-TFMHD). After 24 h, protein samples were run on a 10% acrylamide separating gel in the presence of 2-mercaptoethanol and stained with Coomassie Blue.

It is presumed that the oligomerization of RNase induced by 3-MHD involves the same type of autoxidative-dependent coupling (Scheme 1, R = CH_3) as observed for 2,5-HD. The strong inhibitory effect of NAC on 3-MHD-derived protein cross-linking is attributed to efficient trapping by thiol of the reactive intermediates responsible for autoxidation-dependent pyrrole−pyrrole coupling. However, the only partial inhibition by NAC of cross-linking by 3-TFMHD suggests operation of a different mechanism in this case.

Role of 3-TFMHD- or 3-MHD-Derived Lysine-Based Pyrroles in Protein Cross-Linking. Although we showed that 3-TFMHD-derived pyrroles are resistant to oxidation, our findings that 3-TFMHD is as capable as 3-MHD in inducing RNase cross-linking led us to determine more directly whether the presence of the 3-(trifluoromethyl)pyrrole in a protein matrix may still result in cross-linking. The strategy adopted is shown in Scheme 2.

Paal−Knorr condensation of 3-TFMHD and 3-MHD with 6-aminocaproic acid permits isolation of the corresponding pyrroles **1** and **2**, which were then coupled to RNase lysine residues through direct EDC/sulfo-NHS coupling at 4 °C in buffer under an argon atmosphere.

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Scheme 2

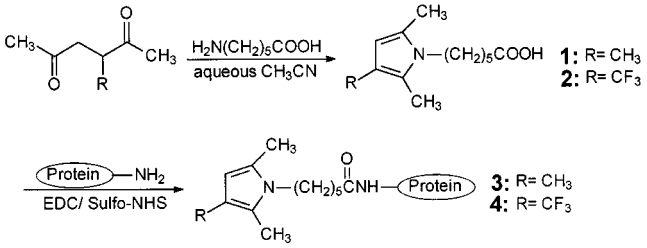

Table 1. TNBS Analysis of Protein Lysine Modification in Modified RNase 3, 4, and 6

	% Lys modified[a]	μmol of Lys modified/ mg of protein[b]
3	62.1[c]	0.45
4	69.9	0.51
6	50.4	0.37

[a] Of TNBS-detected lysine: 100 − 100 × (control OD_{TNBS} − modified OD_{TNBS}), where OD_{TNBS} refers to background-corrected readings (measured at λ = 420 nm). [b] From column 1, assuming 730 nmol of Lys/mg of RNase A (FW = 13 700). [c] Reading was measured for crude protein **3** without dialysis.

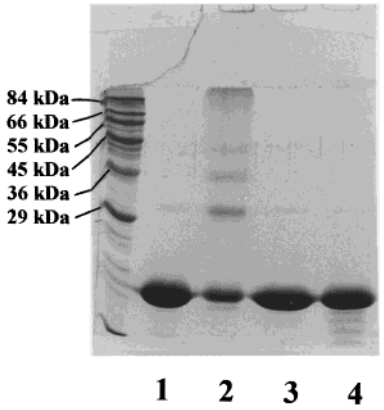

Figure 2. SDS−PAGE analysis of pyrrolated RNase A derivatives following 96 h exposure to air in pH 7.4 phosphate buffer: control RNase A (lane 1), RNase-coupled 6-(2,3,5-trimethylpyrrol-1-yl)caproic acid (**3**) (lane 2), RNase-coupled 6-(2,5-dimethyl-3-(trifluoromethyl)pyrrol-1-yl)caproic acid (**4**) (lane 3), and RNase-coupled 6-(2,5-dimethyl-3-(trifluoromethyl)pyrrol-1-yl)acetic acid (**6**) (lane 4).

The extent of lysine modification was then assessed by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay[21] (Table 1). More than 50% of RNase lysine groups were modified, presumed to reflect mainly tethered pyrrole moieties **3** and **4** (some EDC-derived side products would also be present). During the latter stage of the 12 h period of EDC-mediated coupling of **1** to RNase, coloration appeared in the solution, suggestive of facile oxidation of the trimethylpyrrole mediated by traces of O_2 present, and the sample was directly analyzed by SDS−PAGE without dialysis. In the case of coupling of **2** to RNase, where no color developed, there were two successive 24 h dialyses to remove unbound reagents and a subsequent 96 h incubation in phosphate buffer (pH 7.4) open to atmospheric O_2, prior to analysis by SDS−PAGE. In the case of modified protein **3**, even without intentional exposure to O_2, formation of RNase dimers and higher oligomers was evident (Figure 2, lane 2) along with high molecular weight material that did not enter the running gel. In contrast, no oligomerization was observed for modified protein **4** (lane 3), even with the extended opportunity for O_2-mediated cross-linking. We also tested modified protein **6**, where the 3-TFMHD-derived pyrrole was attached to RNase through a shorter tether (Scheme 3); no dimerization was observed after 96 h aerobic incubation (Figure 2, lane 4).

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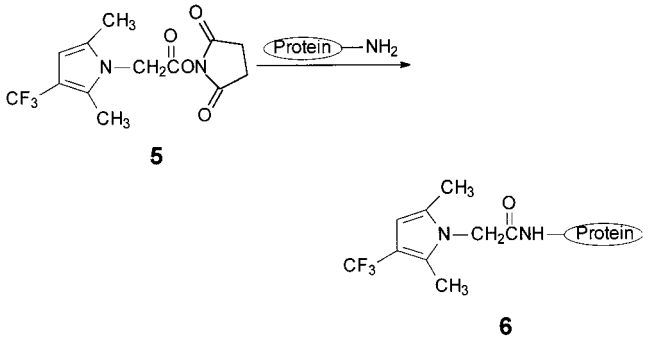
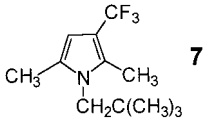
Scheme 3



These experiments demonstrate that the 3-TFMHD-derived pyrrole is not responsible for 3-TFMHD-induced protein cross-linking. In contrast, the 3-MHD-derived pyrrole is clearly susceptible to autoxidation-dependent protein cross-linking, presumably occurring at least in part via the lysine-based pyrrole−pyrrole oxidative coupling[16,18] depicted in Scheme 1.

Structural Elucidation of 3-TFMHD−Neopentylamine Adducts that Could Explain 3-TFMHD-Mediated Protein Cross-Linking. The fact that the 3-TFMHD-derived pyrrole is resistant to autoxidation and cannot account for the protein cross-linking occurring when RNase is modified with 3-TFMHD led us to investigate the reaction of 3-TFMHD with a simple primary amine as a lysine surrogate. Of the various amines used previously,[14] neopentylamine was the amine of choice for the current work based on the simplicity of its NMR spectrum, even though the initial condensation reactions are slower than when sterically unencumbered primary amines are used. The incubation of 3-TFMHD and an excess of neopentylamine in $CDCl_3$, monitored by both ^{1}H NMR and TLC, was previously reported to give rise to two (minor) side products in addition to the expected pyrrole 7.[14] A preparative scale reaction was



now carried out using a 10-fold excess of neopentylamine in acetonitrile−aqueous sodium phosphate pH 7.4 buffer (1:2), followed by workup and silica gel flash chromatographic purification. Under these different reaction conditions, comparison of the ^{1}H NMR spectral integrals of the isolated compounds to the initial crude reaction mixture indicated that about 80% of the 3-TFMHD-containing derived products represented a nearly 1:1 mixture of pyrrole 7 and one of the side products. The second side product was present in 5−10% yield, with additional side products being detectable only by GC−MS. Clearly the switch from $CDCl_3$ to aqueous CH_3CN resulted in favoring the formation of the side products relative to the pyrrole.

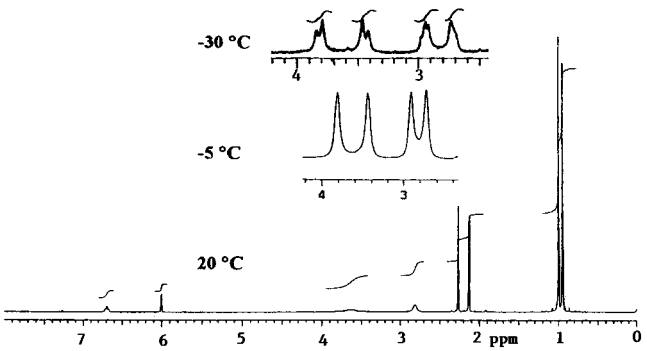


Figure 3. ^{1}H NMR spectra of the 2:1 neopentylamine−3-TFMHD adduct **8**.

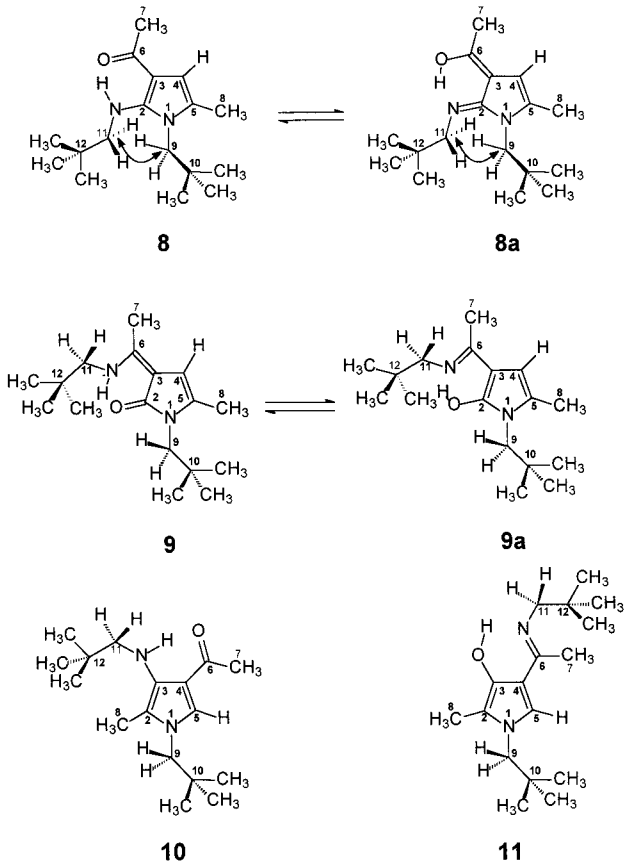
The structure of the major side product was elucidated on the basis of ^{1}H NMR (Figure 3), ^{13}C NMR, IR, 2D rotating frame Overhauser effect spectroscopy (ROESY), 2D total correlation spectroscopy (TOCSY) (see Supporting Information), and electron impact high-resolution mass spectroscopy (HRMS). The ^{1}H NMR spectrum (20 °C, Figure 3) shows two singlets at 0.97 and 1.01 ppm representing two different *tert*-butyl signals, and thus implicating two neopentylamine fragments. The remaining two singlets at 2.13 and 2.27 ppm are assigned as two CH_3 groups from the 3-TFMHD molecule. At 20 °C, the neopentyl methylene signals must then be accounted for by the broad 2H peaks at 2.81 and 3.64 ppm. At −5 °C these broad resonances sharpen to broad doublets, which at −30 °C exhibit complex splittings (vide infra). It was clear that this product represents an adduct of two molecules of neopentylamine and one molecule of 3-TFMHD. The ^{13}C NMR spectrum further confirmed the 2:1 adduct stoichiometry by showing two sets of three *N*-neopentyl carbon signals.

The presence of a trisubstituted pyrrole ring was indicated by both ^{13}C and ^{1}H NMR spectroscopy. There was also an apparent carbonyl group implicated by a lone downfield ^{13}C signal and an IR absorption at 1610 cm^{-1}. This low frequency as well as a long wavelength UV absorbance (λ_{max} = 334 nm) is suggestive of extended conjugation. Our finding that the carbonyl group is resistant to reduction by $NaBH_4$ suggested its possible deactivation by electron-donating groups. Moreover, *no fluorine* atoms could be detected by ^{19}F NMR analysis, despite retention of the CF_3 carbon atom, suggesting a mechanism involving multiple fluoride elimination steps. HRMS analysis revealed a molecular ion peak at 278.2362, corresponding to the molecular formula $C_{17}H_{30}N_2O$.

On the basis of the spectral data and the assumption of maintenance of the original 3-TFMHD carbon skeleton, four possible isomers (**8**−**11**) were initially considered for the major 2:1 adduct (Scheme 4), two of which (**8** and **9**) could exist as two possible tautomers. Of these four structures, **8**/**8a** and **9**/**9a** were primary candidates, because they would preserve the original oxidation state of the CF_3 carbon (position 2). In the case of compounds **10** and **11**, the original CF_3 carbon corresponds to position 5, and generation of these structures would thus require an internal redox reorganization.

In only one case (**8** and **8a**) would the neopentyl CH_2 groups be close enough in space to warrant expectation of a significant NOE effect (indicated by the double-headed arrow). Indeed, the two-dimensional ROESY spectrum displayed cross-peaks between two protons at

Scheme 4



2.73 and 2.92 ppm (9-CH_2) and two protons at 3.45 and 3.81 ppm (11-CH_2). As stated above, the finding that the neopentyl methylenes convert from broad 2H signals at 20 °C to apparent doublets at −5 °C and then to a doublet of doublets (δ 3.64) and a doublet of multiplets (δ 2.81) at −30 °C (Figure 3) is indicative of temperature-dependent *diastereotopism*. This is consistent with structures **8** and **8a**, in which the *tert*-butyl group in the pyrrole *N*-neopentyl fragment is sterically constrained from lying in the plane of the 2,5-disubstituted pyrrole ring, probably as a cooperative consequence of the presence of the exocyclic *N*-neopentyl group (molecular models suggest that the two *tert*-butyl groups project to opposite sides of this plane). The conformational restriction at low temperature prevents the chemical equivalency of the two neopentyl methylene protons on each group.

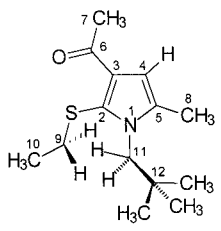
TOCSY was further employed to distinguish between **8** and **8a**. The long-range coupling between the pyrrole ring proton at 6.01 ppm and the 8-C*H*$_3$ (2.13 ppm) as well as coupling between the N*H* and *N*-methylene protons (C-11) in both the TOCSY spectrum and the −30 °C ^{1}H NMR spectrum (Figure 3) are consistent only with **8**.

The structural characterization of the minor side product was also achieved through ^{1}H and ^{13}C NMR, 2D COSY, IR, and HRMS (spectral data are included in the Experimental Section). The ^{1}H and ^{13}C NMR spectra indicated that this product also represented an adduct of two molecules of neopentylamine and one molecule of 3-TFMHD, and HRMS indicated it was an isomer of **8**. Although it was presumed that **8** and **8a** (and **9** and **9a**) would equilibrate during the time scale of isolation, all five structures remaining in Scheme 4 besides **8** were initially considered. Among them, only **9** and **10** could

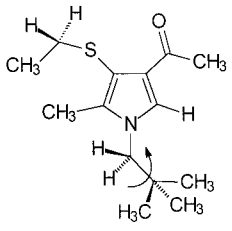
account for the appearance of a C*H*$_2$ doublet at 3.16 ppm and an −N*H* triplet at 5.70 ppm (in $CDCl_3$). The structure of the minor 2:1 adduct was finally assigned as **9** on the basis of the unlikelihood of **10** (and **11**) stated above and the following spectral data: (1) the very upfield carbonyl-like carbon (at 166.12 ppm) is more consistent with the amide moiety in **9** than with the ketone moiety in **10**; (2) the $^1H-^1H$ coupling between the vinyl proton at 5.96 ppm and one C*H*$_3$ group at 2.20 ppm in the COSY spectrum is more consistent with the four-bond coupling in **9** than with the five-bond coupling in **10**.

The effect of the initial ratio of [neopentylamine]$_0$/[3-TFMHD]$_0$ on the formation of these 2:1 adducts was investigated by GC−MS analysis of crude reaction mixtures using isolated samples (**8** and **9**) as internal standards. It was observed that higher [neopentylamine]$_0$/[3-TFMHD]$_0$ ratios led to greater amounts of **8** and **9** relative to the 1:1 pyrrole adduct **7**, as expected, whereas at [neopentylamine]$_0$/[3-TFMHD]$_0$ ratios of ≤1:5, only trace amounts of the 2:1 adducts accompanied pyrrole **7**. Using sterically less hindered primary amines such as *n*-butylamine, NMR spectra of the product mixtures indicated formation of the analogous 2:1 adducts upon incubation with 3-TFMHD in aqueous CH_3CN (data not shown). Another interesting finding was that addition of a large excess of CsF to the reactions using [neopentylamine]$_0$/[3-TFMHD]$_0$ ratios that otherwise yield substantial amounts of 2:1 adducts, led almost exclusively to pyrrole **7**. This suggests that the des-fluoro 2:1 adducts **8** and **9** are formed through a mechanism where at least the first fluoride elimination step is reversible.

Products from Incubation of 3-TFMHD and Neopentylamine in the Presence of Ethanethiol. The significant production of two different 2:1 adducts in the reaction of neopentylamine with 3-TFMHD can explain observed 3-TFMHD-dependent protein cross-linking on the basis of capturing two lysine ϵ-amino groups on separate protein monomers into such adducts. Since the presence of added thiol NAC partially inhibited 3-TFMHD-induced RNase cross-linking, it was of interest to determine the effect of added thiol on the reaction between 3-TFMHD and neopentylamine. The reaction of 3-TFMHD and neopentylamine in aqueous CH_3CN in the presence of ethanethiol was monitored by TLC (EtOAc/hexane 1:4 v/v), and the products were separated by flash chromatography. Besides pyrrole **7** (∼30%) and the 2:1 adducts (**8** ≫ **9**, total 35−40%), a novel thiol−pyrrole conjugation product was isolated (∼30%). Assuming retention of the 3-TFMHD skeleton, only two isomers (**12** and **12a**) would match the observed molecular formula



12



12a

indicated by HRMS and the general NMR patterns (spectral data are included in Experimental Section). As before, only one of these two (**12**) would preserve the oxidation state of the CF_3 carbon (position 2).

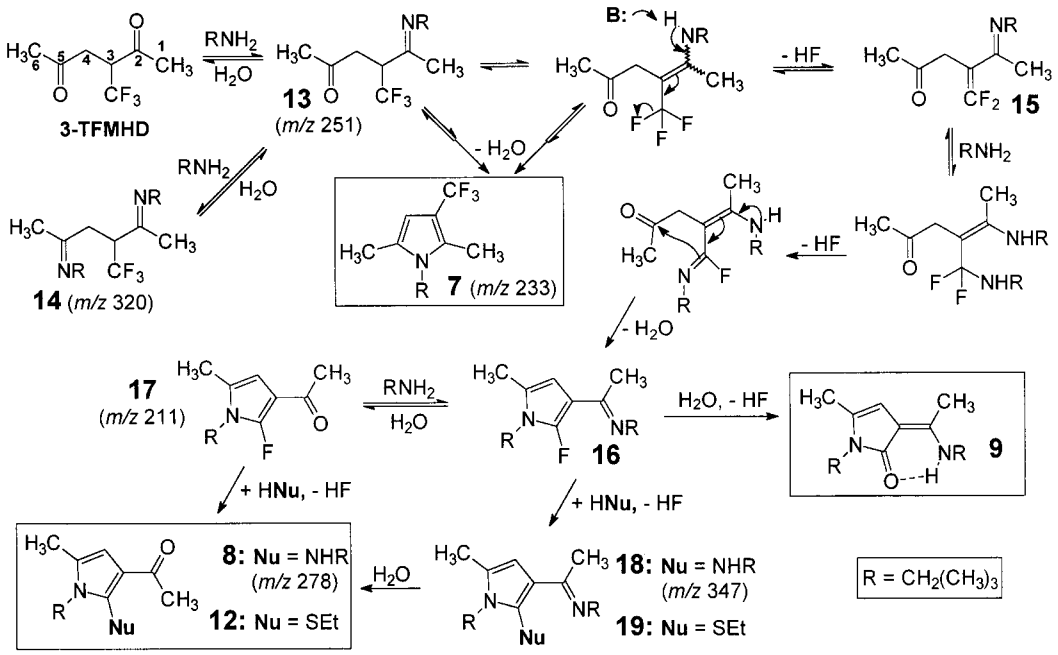
The two *N*-neopentyl methylene protons appeared in the ^1H NMR spectrum as unresolved humps at 3.54 and 4.28 ppm at 25 °C but sharpened to broad doublets at −40 °C. This is indicative of conformationally enforced diastereotopism and is only consistent with the 2,5-disubstituted pyrrole structure in **12**. Simple molecular models reveal that the equilibration of the *N*-neopentyl *tert*-butyl group from above to below the plane is sterically hindered and thus apparently slow on the NMR time scale at lower temperature. The steric constraint in **12** also causes the SCH_2 protons to become diastereotopic (unresolved hump at 2.74 ppm at 25 °C). In contrast, for isomer **12a** there would be free rotation (↶) about the tert-butyl−CH_2 bond, and thus, both N-CH_2 and S-CH_2 signals would be expected to appear as sharp singlets.

The fact that **12** differs from **8** only in that the pyrrole 2-substituent is SEt rather than $NHCH_2C(CH_3)_3$ suggests that there is direct competition between thiol and amine at a crucial intermediate stage of the mechanism. Since **8** is the major amine-derived 2:1 adduct, the implicated competition has two important consequences. First, it suggests that protein cross-linking by 3-TFMHD could involve one lysine residue (contributing the pyrrole *N*) and either one cysteine residue or a second lysine residue as the pyrrole substituent, though the infrequency of free protein Cys residues would suggest a greater likelihood of Lys-Lys than Lys-Cys cross-linking. Second, this competition would explain the ability of NAC to partially inhibit protein cross-linking induced by 3-TFMHD on the basis of a *stoichiometric* nucleophilic competition. This is in contrast to the ability of NAC to totally inhibit cross-linking by 3-MHD (Figure 1), consistent with a *chain-breaking* action of NAC in an autoxidation-dependent radical process. In this latter case, NAC could act as a hydrogen atom donor or by nucleophilic trapping of a pyrrole radical−cation chain carrier. Such mechanistic distinction would explain why NAC, at a given concentration, is much more effective at inhibiting (autoxidation-dependent) cross-linking by 3-MHD than (autoxidation-independent) cross-linking by 3-TFMHD.

Mechanisms of 3-TFMHD-Induced Cross-Linking. To gain information on the mechanism and nature of intermediates leading to the formation of the pyrrole and side products described above, reactions of 3-TFMHD with different stoichiometric ratios of neopentylamine or *n*-butylamine in $CHCl_3$ were monitored over time by GC−MS. In the case of neopentylamine, peaks at *m*/*z* values of 211, 251, 320, and 347 were observed in addition to the *m*/*z* values of 233 (pyrrole **7**) and 278 (2:1 adducts **8** and **9**) representing the isolated products. Over time, the mass peaks at 233 and 278 grew at the expense of the others. Although a detailed analysis of these data was not attempted, a reasonable mechanistic scheme consistent with these observations and the final formation of **7**, **8**, **9**, and (with added ethanethiol) **12** is shown in Scheme 5, where H**Nu** corresponds to either neopentylamine or, when added, ethanethiol.

According to this mechanism, reaction of 3-TFMHD with neopentylamine first forms mono-Schiff base **13** at C-2 (should be more reactive) or at C-5 (not shown), followed by cyclization to form pyrrole **7** (the classical Paal−Knorr condensation).[22] In competition with cyclization would be equilibration of **13** and free amine with

Scheme 5



di-Schiff base **14** seen by GC−MS. In addition, C-2 Schiff base **13** could also, likely through the corresponding enamine, undergo elimination of HF to give β,β-difluoroenimine **15**. Our observation that the presence of a large excess of CsF led to exclusive formation of **7** suggests that the HF elimination step forming **15** is reversible. It is known that trifluoromethyl groups are quite resistant to direct nucleophilic substitution but that substitution can alternatively occur instead through a resonance-assisted elimination-addition sequence, such as we speculate in Scheme 5. There is good literature precedent for nucleophilic substitution at $CF_3CR{=}CR{-}NR_2$ groups proceeding through conjugate fluoride elimination and addition to the resulting β,β-difluoroenimine intermediates.[23−25]

Intermediate **15**, once formed, can then undergo conjugate addition by another molecule of amine, followed by elimination of HF to give fluoropyrrole **16**, proposed to be the direct precursor to product **9** by reaction with water, or to lead to substituted pyrroles **18** (seen by GC−MS) and **19** by reaction with neopentylamine and (when added) ethanethiol, respectively. Pyrrolic imines **18** and **19** are apparently unstable with respect to hydrolysis to isolated compounds **8** and **12**. The latter two compounds could be formed by the alternate sequence of reactions (hydrolysis before nucleophilic substitution), which seems to occur as well, as indicated by GC−MS evidence for the intermediacy of **17**. There is ample precedent for nucleophilic substitution of 2-halopyrroles containing resonance electron-withdrawing groups at C3,[26] and these reactions undoubtedly occur via an addition−elimination sequence. Although more definitive intermediate characterization and kinetics could be employed to determine the likelihood of the pathways shown relative to several other possible routes to the isolated products, we believe that Scheme 5 illustrates at least some of the chemistry occurring in our reactions.

Conclusion. In this paper, we have unveiled a likely explanation for the cross-linking of proteins that occurs by exposure to 3-TFMHD. This was a surprising result for a γ-diketone that was originally studied for the purpose of converting protein-based lysine ϵ-amino side chains to pyrroles that were incapable of the usual autoxidation-dependent cross-linking reaction ascribed to 2,5-hexanedione and its alkylated analogues. These results point to another reminder of the possible elimination−addition outcomes that may occur for trifluoromethyl-substituted compounds of various types. This recognition is particularly important in that the CF_3 group is often used as a presumed unreactive substituent to accomplish isosteric electronic perturbation of candidate drugs and enzyme inhibitors in structure−activity efforts. Although the CF_3 group may be inert in the context of the initial drug, labilization may occur during metabolism of the drug, resulting in intermediates capable of covalent binding through elimination−addition pathways, thus possible giving rise to toxic side effects.

Experimental Section

General. ^{1}H NMR (300 MHz), ^{13}C NMR (75.1 MHz), APT (Attached Proton Test), and ^{1}H−^{1}H COSY spectra were recorded at ambient temperature (20 °C) unless specified otherwise. ROESY and TOCSY spectra were recorded on a 600 MHz instrument. In all cases, tetramethylsilane or the solvent peak served as an internal standard for reporting chemical shifts, which are expressed as ppm downfield from TMS (δ scale). In the ^{13}C NMR line listings, APT designations are given, following the chemical shift, as (+) to indicate secondary and quaternary carbons or (−) to indicate primary and tertiary carbons. Low-resolution GC−MS data were obtained with the mass selective detector set at 70 eV ionization potential and using a HR-I capillary column (5 mm × 0.53 mm). High-resolution mass spectra (HRMS) were obtained at 20 eV. FT-IR spectra were recorded following calibration to the 1602.0 cm^{-1} band of polystyrene. UV spectra were obtained at

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constant temperature maintained using a water-jacketed multiple cell holder. Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} glass plates. Flash column chromatography was performed using 230−400 mesh silica gel. Dialysis was carried out using 14 000 M_r cutoff membrane tubing. Preparations of 3-TFMHD and 3-MHD were described previously.[14] All other materials were reagent grade.

6-(2,3,5-Trimethylpyrrol-1-yl)caproic Acid (1). 3-MHD (0.64 g, 5 mmol), 0.78 g (6 mmol) of 6-aminocaproic acid, and 0.51 g (5 mmol) of triethylamine were dissolved in 20 mL of MeOH−H_2O (3:1 v/v), and the mixture was kept stirring for 10 h under nitrogen at room temperature. Then the reaction mixture was concentrated and extracted with CH_2Cl_2, and the organic layer was dried (Na_2SO_4) and evaporated to give a brown residue that was purified by silica gel chromatography (MeOH−EtOAc, 1:2 v/v as eluent), with fraction collection under N_2, to yield 550 mg (50%). Compound **1** was stored under N_2 at −18 °C: ^{1}H NMR (CD_3COCD_3) δ 1.36−1.46 (2H), 1.55−1.71 (4H), 1.93 (s, 3H), 2.11 (s, 3H), 2.17 (s, 3H), 2.33 (t, J = 7.4 Hz, 2H), 3.75 (t, J = 7.5 Hz, 2H), 5.53 (s, 1H); ^{13}C NMR δ 9.86 (−), 11.42 (−), 12.30 (−), 25.36 (+), 27.04 (+), 31.68 (+), 34.13 (+), 43.87 (+), 107.96 (−), 113.07 (+), 123.11 (+), 125.88 (+), 174.76 (+); MS m/z 223 (M^+), 122 (M − $(CH_2)_4$COOH); HRMS m/z calcd for $C_{13}H_{21}NO_2$ 223.1573, found 223.1580.

Conjugation of 1 onto RNase A. Solutions of EDC (9.8 mg, 0.033 mmol) and sulfo-NHS (6.5 mg, 0.030 mmol) in 10 μL of H_2O each were added successively to a solution of **1** (8.3 mg, 0.030 mmol) in 180 μL of DMF under argon. The resulting solution, after 2 h incubation at room temperature, was added to 800 μL of a solution of RNase (12.5 mg/mL) in 100 mM phosphate buffer (pH 7.2) under argon. All solutions were prebubbled with argon. The mixture was kept at 4 °C for 12 h under argon, during which time a brown coloration developed, apparently owing to traces of O_2 present. Without dialysis, the extent of modification of lysines was determined by the TNBS assay,[21] and the modified protein **3** solution was directly subjected to SDS−PAGE.

6-(2,5-Dimethyl-3-(trifluoromethyl)pyrrol-1-yl)caproic Acid (2). 3-TFMHD (150 mg, 0.82 mmol) was added to solution of 170 mg of 6-aminocaproic acid (1.30 mmol) and 400 mg of CsF in 1:1 (v/v) CH_3CN−H_2O. The reaction mixture was incubated for 24 h under nitrogen at room temperature and then concentrated to give a yellow residue that was purified by silica gel flash chromatography (MeOH−EtOAc, 1:4 v/v) to yield 150 mg (66%): ^{1}H NMR ($CDCl_3$) δ 1.36−1.46 (m, 2H), 1.55−1.71 (m, 4H), 2.16 (s, 3H), 2.24 (s, 3H), 2.35 (t, J = 7.4 Hz, 2H), 3.72 (t, J = 7.5 Hz, 2H), 5.96 (s, 1H), 11.10 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 10.53 (−), 12.21 (−), 24.34 (+), 26.30 (+), 30.43 (+), 33.90 (+), 43.51 (+), 103.99 (−), 109.73 (+, q, $^2J_{C-F}$ = 35.3 Hz), 121.32 (+), 124.96 (+, q, $^1J_{C-F}$ = 264.5 Hz, CF_3), 127.25 (+), 179.71 (+); MS m/z 277 (M^+), 258 (M − F), 176 (M − $(CH_2)_4$COOH); HRMS m/z calcd for $C_{13}H_{18}NF_3O_2$ 227.1290, found 227.1292.

Conjugation of 2 onto RNase A. Solutions of sulfo-NHS (16.2 mg, 0.075 mmol) in 20 μL of H_2O and 29.0 mg (0.098 mmol) of EDC in 30 μL of H_2O were added successively to a solution of **2** (16.7 mg, 0.075 mmol) in 450 μL of DMF. After 2 h incubation under nitrogen at room temperature, the above solution was added to 2 mL of a solution of RNase A (12.5 mg/mL) in 100 mM phosphate buffer, pH 7.2. The mixture was kept at 4 °C for 12 h, followed by two successive 24 h dialyses against 1 L of 100 mM sodium phosphate buffer, pH 7.2, to afford modified protein **4**. The extent of modification of lysines was determined by the TNBS assay.[21]

2-(2,5-Dimethyl-3-(trifluoromethyl)pyrrol-1-yl)acetic Acid *N*-Hydroxysuccinimide Ester (5). Preparation of the required precursor 2-(2,5-dimethyl-3-(trifluoromethyl)pyrrol-1-yl)acetic acid was carried out analogously to that of **2** using glycine instead of aminocaproic acid: ^{1}H NMR ($CDCl_3$) δ 2.14 (s, 3H), 2.23 (s, 3H), 4.53 (s, 2H), 6.03 (s, 1H), 6.39 (br, 1H); MS m/z 221(M^+), 202 (M − F), 176 (M − COOH). The pyrrolated glycine (221 mg, 1 mmol) and 121 mg (1.05 mmol) of *N*-hydroxysuccinimide were dissolved in 15 mL of CH_2Cl_2, followed by addition of 217 mg of *N*,*N*′-dicyclohexylcarbodiimide (1.05 mmol). The mixture was incubated for 4 h, and the precipitated *N*,*N*′-dicyclohexylurea was filtered off and washed twice with CH_2Cl_2. The combined filtrate and washings were evaporated to yield a crude residue, which was purified by silica gel flash chromatography (EtOAc−hexanes, 1:1 v/v as eluent), yielding 250 mg (79%) of **5**: ^{1}H NMR ($CDCl_3$) δ 2.19 (s, 3H), 2.28 (s, 3H), 2.79 (s, 4H), 4.81 (s, 2H), 6.03 (s, 1H); MS m/z 318 (M^+), 299 (M − F), 176 (M − $CO_2N(C_4H_4O_2)$); HRMS m/z calcd for $C_9H_{10}NF_3O_2$ 318.0828, found 318.0821.

Conjugation of 5 onto RNase A. A solution of **5** in DMF (10 μL of 600 mM) was added to 190 μL of RNase A (10.5 mg/mL) in 10 mM sodium phosphate buffer (pH 7.0), and the mixture was incubated for 12 h at room temperature. The mixture was then dialyzed against 500 mL of sodium phosphate buffer (pH 7.0) twice to give modified protein **6**. The extent of lysine modification was determined by the TNBS assay.[21]

Isolation of 2:1 Adducts from the Reaction of Neopentylamine with 3-TFMHD. Neopentylamine (1.74 g, 20 mmol) was dissolved in 25 mL of 1:2 CH_3CN−water, and solid NaH_2PO_4 was added until the pH was lowered to 7.4. To this buffered solution was added 3-TFMHD (0.31 g, 2 mmol), and the reaction mixture was kept stirring for 12 h under nitrogen. The solvent was removed under reduced pressure, and the resulting crude residue was subjected to silica gel flash chromatographic separation (EtOAc−hexanes, 1:1 v/v). Besides the desired pyrrole **7**[14] (186 mg, 40%), two neopentylamine−3-TFMHD 2:1 adducts, **8** and **9**, were isolated. **8** (239 mg, 43%): ^{1}H NMR ($CDCl_3$, 20 °C) δ 0.97 (s, 9H), 1.01 (s, 9H), 2.13 (s, 3H), 2.27 (s, 3H), 2.81 (br, 2H), 3.64 (br, 2H), 6.01(s, 1H), 6.69 (t, J = 7.4 Hz, 1H); ^{1}H NMR ($CDCl_3$, −5 °C) δ 0.97 (s, 9H), 1.01 (s, 9H), 2.13 (s, 3H), 2.27 (s, 3H), 2.68 (br, 1H), 2.90 (br, 1H), 3.46 (br, 1H), 3.80 (br, 1H), 6.01 (s, 1H), 6.78 (br, 1H); ^{1}H NMR ($CDCl_3$, −30 °C) δ 0.97 (s, 9H), 1.01 (s, 9H), 2.13 (s, 3H), 2.27 (s, 3H), 2.73 (d, J = 15.0 Hz, 1H), 2.92 (d, J = 15.0 Hz, 1H), 3.45 (d, J = 15.0 Hz, 1H), 3.81 (d, J = 15.0 Hz, 1H), 6.01 (s, 1H), 6.83 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 13.51 (−), 27.14 (−), 27.45 (−), 28.53 (−), 32.07 (+), 35.52 (+), 53.89 (+), 59.99 (+), 106.11 (−), 107.85 (+), 124.18 (+), 150.26 (+), 192.90 (+); HRMS m/z calcd for $C_{17}H_{30}N_2O$ 278.2360, found 278.2362; IR (KBr) ν (cm^{-1}) 3452, 2954, 1610, 1578, 1478, 1326; UV (MeOH) λ_{max} = 334 nm. **9** (33 mg, 6%): ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 9H), 0.95 (s, 9H), 2.20 (s, 3H), 2.51 (s, 3H), 3.16 (d, J = 3.2 Hz, 2H), 3.59 (s, 2H), 5.70 (t, J = 3.20 Hz, 1H), 5.96 (s, 1H);^{13}C NMR ($CDCl_3$) δ 12.99 (−), 13.64 (−), 27.33 (−), 28.68 (−), 32.03 (+), 34.77 (+), 50.04 (+), 53.94 (+), 104.65 (−), 114.09 (+), 128.63 (+), 134.08 (+), 166.12 (+); HRMS m/z calcd for $C_{17}H_{30}N_2O$ 278.2360, found 278.2350; IR (KBr) ν (cm^{-1}) 3330, 2958, 1634, 1580, 1536, 1478. 2D COSY spectra show that the downfield peak at 5.96 ppm is coupled with one CH_3 peak at 2.20 ppm and that the NH triplet at 5.70 ppm is coupled with the doublet at 3.16 ppm.

Reactions of 3-TFMHD with Amines in $CDCl_3$. A chloroform-d_3 solution (0.5 mL in NMR tube) containing 0.022 mmol of 3-TFMHD and either 0.0073, 0.022, 0.066, or 0.33 mmol of neopentylamine was incubated under N_2. The ^{1}H NMR spectrum was recorded at 48 h, then 1.5 μL of each reaction mixture was analyzed by GC−MS. The relative ratio of pyrrole **7** (t_R = 5.6 min, m/z 233) to the adducts **8** and **9** (t_R = 9.0 min, m/z 278) was calculated on the basis of the area of ion abundance from GC−MS spectra, using the isolated compounds (above) as internal references.

Reaction of 3-TFMHD with Neopentylamine and Ethanethiol in Aqueous Acetonitrile. 3-TFMHD (80 mg, 0.44 mmol) was added into 10 mL of 50% aqueous CH_3CN containing 0.51 g of neopentylamine (6.55 mmol) and 0.50 g of ethanethiol (8.06 mmol). The solution was kept stirring under N_2 for 48 h and then evaporated, and the residue was subjected to preparative TLC (EtOAc/hexane 1:4 v/v as eluent). Besides pyrrole **7** (31 mg, 30%) and adducts **8** (43 mg, 35%) and **9** (6 mg, 5%), 33 mg of **12** (30%) was isolated: ^{1}H NMR ($CDCl_3$, 20 °C) δ 0.93 (s, 9H), 1.05 (t, J = 7.4 Hz, 3H), 2.25 (s, 3H), 2.49 (s, 3H), 2.73 (br, 2H), 3.54 (br, 1H), 4.28 (br, 1H), 6.41 (s, 1H); ^{1}H NMR ($CDCl_3$, −40 °C) δ 0.93 (s, 9H), 1.05 (t, J = 7.4 Hz, 3H), 2.25 (s, 3H), 2.49 (s, 3H), 2.64 (br, 1H), 2.78 (br, 1H), 3.51 (br d, J = 15.0 Hz, 1H), 4.32 (br d, J = 15.0 Hz,

1H), 6.41 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.19 (−), 14.46 (−), 28.56 (−), 31.66 (+), 34.68 (+), 53.92 (+), 110.80 (−), 127.06 (+), 128.06 (+), 131.72 (+), 193.74 (+); HRMS *m*/*z* calcd for $C_{14}H_{23}NOS$ 253.1502, found 253.1508.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **1** and **2**; ^{1}H NMR spectrum of **5**; ^{13}C NMR, ROESY, and TOCSY spectra of **8**; ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, and IR spectra of **9**; ^{1}H NMR (ambient temperature and −40 °C) and ^{13}C NMR spectra of **12**. This materials is available free of charge via the Internet at http://pubs.acs.org.

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