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Design of Radical-Resistant Amino Acid Residues: A Combined Theoretical and Experimental Investigation

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Abstract: Ab initio calculations have been used to design radical-resistant amino acid residues. Optimized structures of free and protected amino acids and their corresponding α-carbon-centered radicals were determined with B3-LYP/6-31G(d). Single-point RMP2/6-31G(d) calculations on these structures were then used to obtain radical stabilization energies, to examine the effect of steric repulsion between the side chains and amide carbonyl groups on the stability of α-carbon-centered peptide radicals. Relative to glycine, the destabilization for alanine and valine residues was found to be approximately 9 and 18 kJ mol^{-1}, respectively, which correlates with the reactivity of analogous amino acid residues in peptides toward hydrogen atom abstraction in conventional free radical reactions. To design amino acid residues that would resist radical reactions, strategies by which the steric effects could be magnified were considered. This resulted in the identification of *tert*-leucine and 3,3,3-trifluoroalanine as suitable molecules. With these amino acid residues, the destabilization of the α-carbon-centered radicals relative to that of glycine is increased substantially to approximately 36 and 41 kJ mol^{-1}, respectively. The theoretical predictions have been supported by experimental observations: a *tert*-leucine derivative was shown to be very slow to react with *N*-bromosuccinimide, while the corresponding trifluoroalanine derivative was found to be inert.

Introduction

Amino acid and peptide radicals have been implicated in a wide variety of biochemical processes and physiological disorders,[1−6] including arteriosclerosis[4] and aging.[5,6] At a molecular level, they are associated with protein damage[7] and enzyme function.[8−18] As a result, studies of their properties are both important and topical. In peptides and proteins, α-carbon-centered radicals (Figure 1) form preferentially,[19−24] because they are extensively stabilized through resonance. Knowledge of other factors affecting the formation of these radicals is fundamental to understanding the basis of their effects.

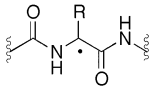

Figure 1. α-Carbon-centered radicals formed from peptides, proteins, and other amino acid derivatives.

Of the α-carbon-centered radicals produced from peptides and other amino acid derivatives, glycyl radicals are the most common and form selectively. This has been observed in EPR and radiolysis studies.[3,25,26] It has also been seen and exploited in the regioselective photoalkylation of peptides and proteins[22−24] and in the bromination of peptides and other amino acid derivatives.[19−21,27,28] Glycyl radicals are also intermediates in enzymic processes. Backbone glycyl radicals are thought to be involved in the catalysis displayed by pyruvate formate lyase,[11,12]

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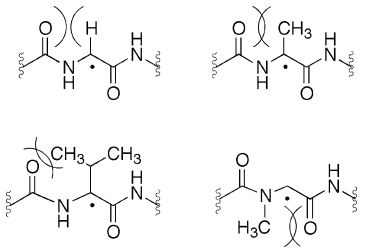
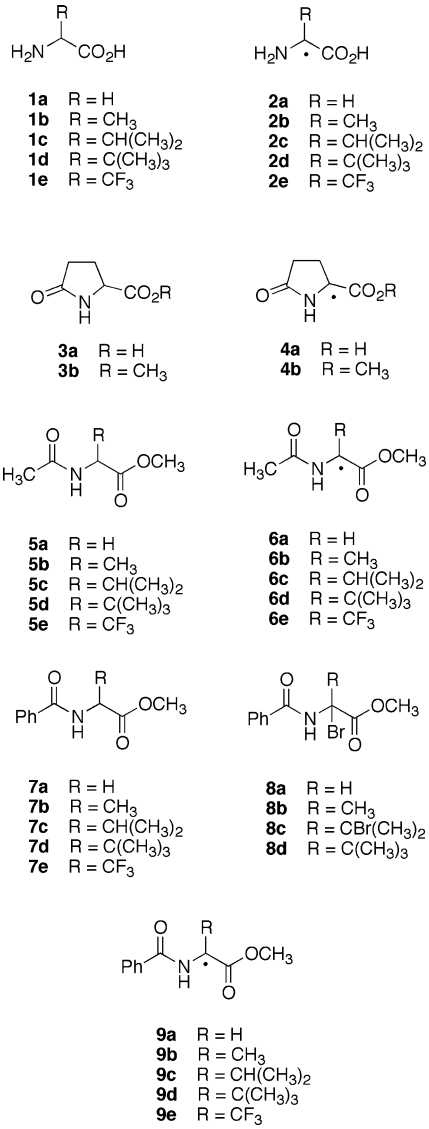


Figure 2. Steric effects associated with planar conformations of peptide radicals.

ribonucleotide reductase,[13] benzylsuccinate synthase,[15] and hexylsuccinate synthase.[16] In addition, the production of peptide hormones by oxidative degradation of their glycine-extended precursors, catalyzed by peptidylglycine α-amidating monooxygenase, proceeds via the corresponding glycyl radicals.[17,18] Model systems for this enzyme also exhibit selectivity for reaction of glycine derivatives.[29−31] Furthermore, selective formation of glycyl radicals is implicated in the oxidation of β-amyloid proteins that are associated with Alzheimer's disease and Jacob-Creuzfeld disease.[32] This oxidation may play a role in neurodegeneration.[32,33]

Several explanations have been given for the predominant formation of glycyl radicals.[19,27,32,34−36] In hydrogen atom abstraction reactions, it has been postulated that they form selectively because they are able to adopt planar conformations which are relatively free of steric interactions.[19,27] By comparison, the α-carbon-centered radicals of other proteinogenic amino acid residues form less readily because they are destabilized as a result of buttressing of their side chains with their amide carbonyls (Figure 2). By analogy, the α-carbon-centered radicals of sarcosine derivatives are less stable than those of glycine residues due to steric interactions associated with the methyl groups.[27,37] An alternative proposal is that glycyl radicals are commonly formed through oxidative side-chain cleavage of amino acid derivatives.[34,35] Intermediate hydroperoxides give rise to alkoxy radicals, which afford α-carbon-centered radicals through β-scission.[34,35,38] Yet another suggestion is that, in peptides and proteins, amino acid residues other than glycine are less exposed and more conformationally constrained, thus limiting radical formation.[32,36]

In light of these various explanations, we sought to quantify the relative stability of glycyl and other α-carbon-centered amino acid radicals and thereby evaluate the effect of the steric interactions illustrated in Figure 2 on the formation of these species. To accomplish this task, we used quantum chemical procedures, which now provide a reliable means of directly examining such factors.[39,40] Rauk and co-workers[41,42] have used a similar approach to study the α-carbon-centered radicals of a range of *N*-formylamino acid amides. However, their objective was quite different from that of the present study, and they did not make specific comparisons with the radicals of the corresponding free amino acids, as we required to assess the steric effects. The systems that we chose for study were glycine, alanine, and valine, due to the variation in bulk of their side chains. The free amino acids **1a**−**c** and their *N*-acetyl methyl ester derivatives **5a**−**c** were investigated, the latter as models for amino acid residues incorporated in peptides. Theoretical methods provide a particularly valuable means of studying the free amino acids, which are otherwise only accessible as the nonzwitterionic species in the gas phase. Pyroglutamic acid **3a** and methyl pyroglutamate **3b** were included in our investigation, as they are known to be peculiarly reactive toward hydrogen-atom transfer.[27]



The results of these initial studies led us to consider strategies by which we could magnify the steric effects, to design amino

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acid residues that would be resistant to radical reactions. Consequently we have also examined *tert*-leucine **1d** and 3,3,3-trifluoroalanine **1e** and their derivatives **5d** and **5e**. Conventional free radical reactions using *N*-bromosuccinimide (NBS) were carried out to complement the calculations and the results of the theoretical and experimental studies were found to be entirely in accord.

Theoretical and Experimental Procedures

Computational Details. Standard ab initio molecular orbital theory and density functional theory calculations were performed with Gaussian 94[43] and molpro 96.[44] For the larger systems, preliminary conformational work was carried out at AM1 using the Spartan and MacSpartan Plus programs, to select the most appropriate conformations. The calculated results correspond to isolated molecules in the gas phase.

Optimized structures and harmonic vibrational frequencies were determined at the B3-LYP/6-31G(d) level. The minimum energy conformers of all the molecules were found to have C_1 symmetry. They were confirmed to be true local minima by means of the vibrational frequency calculations that showed all the frequencies to be real. Zero-point vibrational energies were obtained by scaling the B3-LYP/ 6-31G-(d) values by 0.9806.[45] Single-point RMP2/6-31G(d) calculations on the B3-LYP/6-31G(d) structures [RMP2/6-31G(d)//B3-LYP/6-31G(d)] were used to obtain improved relative energies. This approach has been found in previous work to produce radical stabilization energies (RSEs) of useful reliability.[39]

The radical stabilization energies were calculated as the energy change in the isodesmic reaction shown in eq 1:

$$R^{\bullet} + CH_4 \rightarrow RH + CH_3^{\bullet} \qquad (1)$$

The RSEs correspond to the differences between the C−H bond dissociation energies (BDEs) of methane and RH[39,40] and reflect the stability of $R^{\bullet}$ compared with $CH_3^{\bullet}$. Our reported RSEs correspond to values at 0 K. Temperature corrections were not applied since they should show substantial cancellation for such reactions.[41] Details of the theoretical results are provided as Supporting Information.

Chemicals. The amino acid derivatives **7c**−**e** were prepared and treated with NBS using standard procedures.[27,46−51] Details are provided as supporting information.

Results

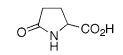
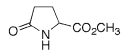
The optimized structures of the amino acids and derivatives **1a**−**e**, **3a**,**b**, and **5a**−**e**, and the corresponding radicals **2a**−**e**, **4ab**, and **6a**−**e**, are illustrated in Figures 3 and 4. The RSEs for the radicals **2a**−**e**, **4a**,**b**, and **6a**−**e** and related species are presented in Tables 1 and 2.

Table 1. Radical Stabilization Energies [0 K, RMP2/6-31G(d), kJ mol^{-1}] of Substituted Methyl Radicals

$XC^{\bullet}RY + CH_4 \rightarrow XCHRY + CH_3^{\bullet}$			
X	Y	R	RSE
H	H	CH_3	13.2[a]
H	H	CF_3	7.5[a]
NH_2	H	H	44.7[a]
AcNH	H	H	37.4
H	CO_2H	H	21.0[a]
H	CO_2CH_3	H	21.6[a]
NH_2	H	CH_3	49.1[a]
NH_2	H	CF_3	41.6
H	CO_2H	CH_3	42.2
H	CO_2H	CF_3	14.2

[a] These data are consistent with literature values.[40,41]

Table 2. Radical Stabilization Energies [0 K, RMP2/6-31G(d), kJ mol^{-1}] of Amino Acid Radicals

$XNHC^{\bullet}RCO_2Y + CH_4$	$XNHCHRCO_2Y + CH_3^{\bullet}$				RSE
$XNHCHRCO_2Y$	$XNHC^{\bullet}RCO_2Y$	X	Y	R	
1a[a]	**2a**[a]	H	H	H	95.9[b]
1b[a]	**2b**[a]	H	H	CH_3	103.4[b]
1c[a]	**2c**	H	H	$CH(CH_3)_2$	98.5
1d	**2d**	H	H	$C(CH_3)_3$	95.3
1e	**2e**	H	H	CF_3	98.3
3a	**4a**				95.1
3b	**4b**				93.3
5a	**6a**	Ac	CH_3	H	82.2
5b	**6b**	Ac	CH_3	CH_3	80.6
5c	**6c**	Ac	CH_3	$CH(CH_3)_2$	67.0
5d	**6d**	Ac	CH_3	$C(CH_3)_3$	45.5
5e	**6e**	Ac	CH_3	CF_3	43.7

[a] Optimized structures are consistent with global minima reported for similar levels of theory.[52−55] [b] These data are consistent with BDEs reported for similar levels of theory.[53]

The free radical reactions of the amino acid derivatives **7a**−**c** with NBS in carbon tetrachloride to give the corresponding bromides **8a**−**c** have been reported.[27,51] Similar treatment of the *tert*-leucine derivative **7d** afforded the bromide **8d**, but the trifluoroalanine derivative **7e** was inert. In competitive experiments using limited quantities of NBS and mixtures of the valine derivative **7c** and either the *tert*-leucine derivative **7d** or the trifluoroalanine derivative **7e**, only the valine derivative **7c** reacted. On the basis of these experiments and literature data for the reactivity of **3b** and **7a**−**c**,[27] the relative rates of reaction of **3b** and **7a**−**e**, to form the corresponding radicals **4b** and **9a**−**e**, were determined (Table 3).

Discussion

Captodative Stabilization. The RSEs calculated in the present work for the ethyl, 2,2,2-trifluoroethyl, aminomethyl, carboxymethyl, carbomethoxymethyl, 1-aminoethyl, glycyl **2a,** and alanyl **2b** radicals (Tables 1 and 2) are consistent with literature data.[40,41,53] The glycyl radical **2a** has an RSE of 95.9 kJ mol^{-1}, indicating that in this species the amino and carboxy groups provide a synergistic stabilization that is 30.2 kJ mol^{-1}

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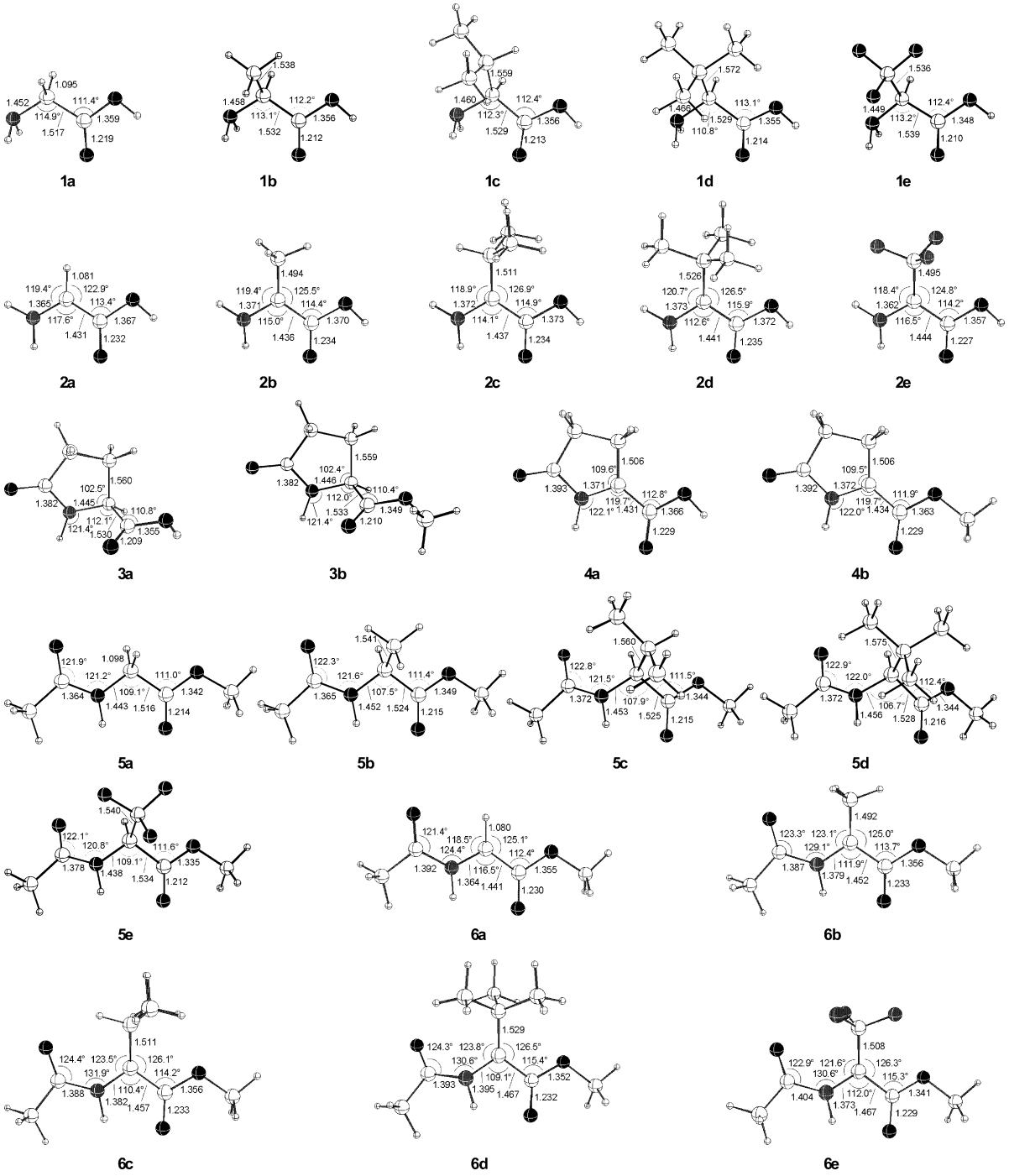

Figure 3. Optimized structures [B3-LYP/6-31G(d)] of the amino acids and derivatives **1a**−**e**, **3a,b,** and **5a**−**e**, and the corresponding radicals **2a**−**e**, **4a,b,** and **6a**−**e**. Bond lengths are in angstroms.

more than the sum of their individual contributions to the RSEs of the carboxymethyl (21.0 kJ mol^{-1}) and aminomethyl (44.7 kJ mol^{-1}) radicals. A similar effect is apparent with the alanyl radical **2b**, where the RSE of 103.4 kJ mol^{-1} is 24.5 kJ mol^{-1} higher than the combined RSEs of the ethyl (13.2 kJ mol^{-1}), aminomethyl, and carboxymethyl radicals. This is consistent with the captodative effect previously reported for similar systems.[52,56,57]

Relative Stability of Glycyl Radicals. The RSE of the glycyl radical **2a** is less than those of the alanyl and valyl radicals **2b** and **2c**, which is expected because the latter are more substituted. The valyl radical **2c** has an RSE lower than that of the alanyl radical **2b**. Presumably this reflects conformational effects associated with the more bulky side chain of the former. Formation of each of the radicals **2b** and **2c** is accompanied by

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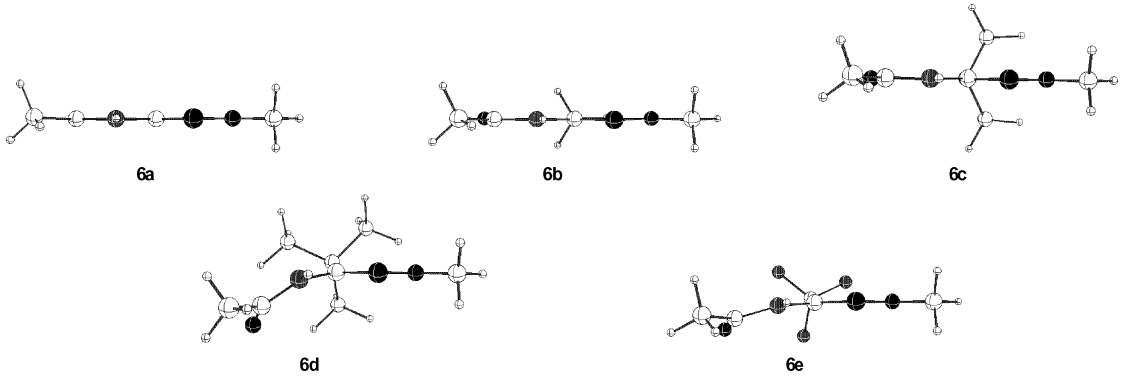

Figure 4. Optimized structures of the radicals **6a**−**e**, viewed from within the plane of the $C^{\alpha}-CO_2$ group.

Table 3. Relative Rates of Reaction of the Amino Acid Derivatives **3b** and **7a**−**e** with *N*-Bromosuccinimide

compd	rel rate of reactn	compd	rel rate of reactn
3b	3.1[a]	**7c**	0.04[a]
7a	1[b]	**7d**	<0.0004
7b	0.33[a]	**7e**	<0.0004

[a] Data from ref 27. [b] Assigned as unity.

a rotation of the amino group, so that the amino hydrogens become coplanar with the α-carbon and the carboxy group. While this change in geometry is readily accommodated in the alanyl system, with valine the isopropyl group must as a consequence rotate away from the closer amino hydrogen to reduce the unfavorable steric interaction that would otherwise ensue (Figure 3).

The RSE of the *N*-acetylglycyl radical **6a** is less than that of the glycyl radical **2a** by 13.7 kJ mol^{-1}. This is consistent with the effect of the acyl group on the RSEs of the aminomethyl (44.7 kJ mol^{-1}) and acetamidomethyl (37.4 kJ mol^{-1}) radicals and can be attributed to the decreased π-electron-donating ability of the acetamido group compared with an amino group. The RSEs of the *N*-acetylalanyl and -valyl radicals **6b** and **6c** are also less than those of the alanyl and valyl radicals **2b** and **2c**, respectively, but the differences in these systems are much greater, being 22.8 and 31.5 kJ mol^{-1}. This is a reflection of the fact that while the RSEs of the alanyl and valyl radicals **2b** and **2c** are 7.5 and 2.6 kJ mol^{-1} greater than that of the glycyl radical **2a**, those of the *N*-acetylalanyl and -valyl radicals **6b** and **6c** are 1.6 and 15.2 kJ mol^{-1} less than that of the *N*-acetylglycyl radical **6a**. Thus, whereas the side chains of the radicals **2b** and **2c** provide additional stabilization, those of the protected derivatives **6b** and **6c** decrease the RSEs.

This destabilization of **6b** and **6c** relative to **6a** is directly attributable to steric interactions between the amide carbonyl groups and the side chains of **6b** and **6c**. This is evident from the optimized structures of these species. The radicals **6a**−**c** prefer a planar arrangement of bonds at their α-carbons (Figure 4), to maximize overlap of their singly occupied p-orbitals with the π-orbitals of the amido and carboxy substituents. However, as the side chain becomes larger, on going from a hydrogen, to a methyl, and then to an isopropyl group, the magnitude of the steric interactions associated with this planar arrangement increases. This is manifest as an enlargement in the O═C−N and $C(O)-N-C^{\alpha}$ bond angles, from 121.4° and 124.4° for **6a**, to 123.3° and 129.1° for **6b**, and 124.4° and 131.9° for **6c** (Figure 3). By contrast with the destabilization of the radicals **6b** and **6c** resulting from the interactions between their side chains and amide carbonyls, such interactions are absent in the minimum energy conformations of the pyroglutamate derivatives **4a** and **4b**. As a result, their RSEs are greater than that of the *N*-acetylglycyl radical **6a**, by 12.9 and 11.1 kJ mol^{-1}, respectively, as is typical of more highly substituted species.

Among the proteinogenic amino acids, valine is representative of those with the most bulky side chains, and with this amino acid residue the steric interactions appear to destabilize the corresponding α-carbon-centered radical by approximately 18 kJ mol^{-1}. This number is based on the difference of 17.8 kJ mol^{-1} between the relative RSEs of the valine derivatives **2c** and **6c** (31.5 kJ mol^{-1}) and those of the glycine derivatives **2a** and **6a** (13.7 kJ mol^{-1}). Equivalently, the isopropyl substituent leads to an increase in the RSE of 2.6 kJ mol^{-1} in going from **2a** to **2c** but a decrease of 15.2 kJ mol^{-1} in going from **6a** to **6c**, a combined difference of 17.8 kJ mol^{-1}. The destabilization will be less with the majority of the other proteinogenic amino acid residues because they are less hindered at the β-position. Accordingly, with alanine the destabilization is of the order of 9 kJ mol^{-1} based on the relative RSEs of **2a**, **2b**, **6a,** and **6b**.

Steric effects analogous to those discussed above and illustrated in Figure 2 are likely to be an important component of the reactivity of amino acid residues in peptides and proteins. They are not of sufficient magnitude to account on their own for the predominant formation of glycyl radicals discussed in the Introduction, and other factors already mentioned are also likely to play significant roles. Nevertheless, there is a strong correlation between the steric effects, illustrated by the RSEs of the radicals **4b** and **6a**−**c** and the rates of hydrogen-atom transfer from the amino acid derivatives **3b** and **7a**−**c**, as reflected in their rates of bromination (Table 3). Thus, the pyroglutamate derivatives **4b** and **3b** have the highest RSE and show the fastest rate of bromination, respectively. The next in the series are the glycine derivatives **6a** and **7a**, then the alanine derivatives **6b** and **7b**, with the valine derivatives **6c** and **7c** having the lowest RSE and being the least reactive.

Radical-Resistant Amino Acid Residues. Examination of the RSEs and minimum-energy conformations of the radicals **6a**−**c** suggested to us that, by exacerbating the interactions between the side chains and the amide carbonyl groups of amino acid derivatives, less stable radicals might be identified. If they were sufficiently less stable, the corresponding amino acid derivatives would be resistant to radical formation. In the minimum energy conformation, the methyl groups of the isopropyl side chain of the valine derivative **6c** are constrained away from the amide carbonyl group so as to minimize

unfavorable interactions. It was envisaged that this would not be possible for all three methyl groups of the *tert*-butyl side chain of a *tert*-leucine derivative and that the corresponding *tert*-leucyl radical would therefore be less stable. In addition, as an alternative means of creating increased unfavorable interactions, it was considered that replacing the hydrogens of the side chain of the alanyl radical **6b** with fluorines would lead to greater electrostatic repulsion of the amide carbonyl, and that 3,3,3-trifluoroalanyl radicals would therefore also be of interest. To examine these hypotheses, the *tert*-leucine and trifluoroalanine derivatives **1d**,**e**, **2d**,**e**, **5d**,**e**, **6d**,**e,** and **7d**,**e** were studied.

The RSE of the *tert*-leucyl radical **2d** is only 3.2 kJ mol^{-1} less than that of the valyl radical **2c**, so the added bulk of the *tert*-butyl side chain has little effect on radical stability for the free amino acids. The trifluoroalanyl radical **2e** has an RSE 5.1 kJ mol^{-1} less than that of the alanyl radical **2b**. To understand the magnitude of this difference, it is informative to consider the effect of fluorine in related systems (Table 1). 2,2,2-Trifluoroethyl radical is 20.7 kJ mol^{-1} less stable than ethyl radical, due to the electron-withdrawing effect of the fluorines. Their effect is enhanced by substitution with an electron-withdrawing carboxy group, as shown by the difference of 26.0 kJ mol^{-1} between the RSEs of 1-carboxyethyl radical (42.2 kJ mol^{-1}) and 1-carboxy-2,2,2-trifluoroethyl radical (14.2 kJ mol^{-1}). By contrast, their effect is reduced to 7.5 kJ mol^{-1} by the amino substituent of 1-aminoethyl radical and 1-amino-2,2,2-trifluoroethyl radical. Presumably the electron-donating amino substituents of 1-amino-2,2,2-trifluoroethyl radical and the trifluoroalanine derivative **2e** partially compensate for the effect of the fluorines.

While the RSEs of the free amino acid radicals **2a**, **2d,** and **2e** all lie within a 3 kJ mol^{-1} range, those of the protected *tert*-leucine and trifluoroalanine derivatives **6d** and **6e** are less than that of the analogous glycine derivative **6a**, by 36.7 and 38.5 kJ mol^{-1}, respectively. The radicals **6d** and **6e** are even 21.5 and 23.3 kJ mol^{-1} less stable than the corresponding valyl radical **6c**. Substituting methyl for trifluoromethyl, or *tert*-butyl for isopropyl, has little effect on the RSEs with the free amino acid radicals **2b**−**e** but reduces the RSEs with the protected amino acid radicals **6b**−**e**, by more than 20 kJ mol^{-1}, in each case. The optimized strucures of the radicals **6d** and **6e** (Figure 4) show that this is due to interactions between the amino acid side chains and their amide carbonyl groups, as predicted. For the radicals **6a**−**c**, planar conformations are adopted, which are distorted within the plane to accommodate strain in the cases of the alanine and valine derivatives **6b** and **6c**. On the other hand, the strain is too great with the *tert*-leucine and trifluoroalanine derivatives **6d** and **6e** and, as a result, the amide is distorted from planarity in each case and twisted out of the plane of the α-carbon and methoxycarbonyl group. This disrupts conjugation and decreases radical stability. When the structure of the radical **6d** was determined with the amido and carboxy groups and the α-carbon constrained to be coplanar, the RSE was calculated to be 4.8 kJ mol^{-1} less than that of the fully optimized structure. In the restricted system, strain is accommodated by increases in the O═C—N and C(O)—N—C$^{\alpha}$ bond angles. While these were found to be 121.4° and 124.4° for the glycine derivative **6a** and 124.4° and 131.9° for the valine derivative **6c**, for the *tert*-leucine derivative **6d** they expand to 125.9° and 137.2°, respectively.

Consistent with the expectation based on the RSEs of the radicals **6d** and **6e**, the corresponding *N*-benzoylamino acid esters **7d** and **7e** are resistant to free radical bromination. Under vigorous conditions, the *tert*-leucine derivative **7d** reacted but the trifluoroalanine derivative **7e** was inert. Both **7d** and **7e** were found to be at least 2 orders of magnitude less reactive than the valine derivative **7c** and at least 3 orders of magnitude less reactive than the glycine derivative **7a** (Table 3).

Conclusion

In summary, it appears that steric repulsion between the amide carbonyl groups and the side chains of proteinogenic amino acid residues decreases the stability and ease of formation of α-carbon-centered peptide radicals. The effects are particularly large with *tert*-leucine and 3,3,3-trifluoroalanine, and as a result, these amino acid residues are resistant to radical formation. They are therefore likely to be useful for incorporation into peptides, to limit oxidative radical degradation, and as alternatives to valine and other proteinogenic amino acids, as chiral auxiliaries in asymmetric free radical reactions. We are currently investigating their application as substitutes for the *C*-terminal glycine residue in mammalian prohormones, where the prevention of radical cleavage of the *C*-terminal amino acid has potential in the regulation of the overproduction of peptide hormones.

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Supporting Information Available: Experimental details for the synthesis of compounds **7c**−**e** and individual and competitive reactions of compounds **7c**−**e** with NBS; Gaussian archive entries for RMP2/6-31G(d)//B3-LYP/6-31G(d) calculations for **1a**−**e**, **2a**−**e**, **3a**,**b**, **4a**,**b**, **5a**−**e**, and **6a**−**e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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