Unexpected Hydrolytic Instability of N-Acylated Amino Acid Amides and Peptides


†Department of Chemistry and Chemical Biology, Indiana University Purdue University Indianapolis, Indianapolis, Indiana 46202, United States
‡Howard University, Washington, DC 20059, United States
§Morehouse College, Atlanta, Georgia 30314, United States

Supporting Information

ABSTRACT: Remote amide bonds in simple N-acyl amino acid amide or peptide derivatives 1 can be surprisingly unstable hydrolytically, affording, in solution, variable amounts of 3 under mild acidic conditions, such as trifluoroacetic acid/water mixtures at room temperature. This observation has important implications for the synthesis of this class of compounds, which includes N-terminal-acylated peptides. We describe the factors contributing to this instability and how to predict and control it. The instability is a function of the remote acyl group, R2CO, four bonds away from the site of hydrolysis. Electron-rich acyl R2 groups accelerate this reaction. In the case of acyl groups derived from substituted aromatic carboxylic acids, the acceleration is predictable from the substituent’s Hammett σ value.

N-Acyl dipeptides are also hydrolyzed under typical cleavage conditions. This suggests that unwanted peptide truncation may occur during synthesis or prolonged standing in solution when dipeptides or longer peptides are acylated on the N-terminus with electron-rich aromatic groups. When amide hydrolysis is an undesired secondary reaction, as can be the case in the trifluoroacetic acid-catalyzed cleavage of amino acid amide or peptide derivatives 1 from solid-phase resins, conditions are provided to minimize that hydrolysis.

INTRODUCTION

In preliminary work generalizing our earlier synthesis1 of N-acylated unnatural amino acid amides 1 to procedures compatible with a wide variety of R2 groups, an unexpected R2-dependent hydrolytic instability was encountered (Scheme 1). During the trifluoroacetic acid (TFA) cleavage of the final Rink resin-bound intermediate 2 to give 1, varying amounts of carboxylic acid 3 were produced.

Scheme 1. Unexpected Presence of Acid 3 in the Cleavage of Resin-Bound Amide 2

It was of concern that the instability of 1 (R3 = H in the encountered case) could be a more general phenomenon and potentially affect the synthesis of N-acylated natural and unnatural amino acid amides, peptides, and proteins that are targets of organic and peptide chemists. Because of these wider implications, we sought to understand the origins and nature of this instability, to develop predictive tools to anticipate it, and to establish reaction and workup conditions to minimize it.

Received: February 4, 2014
Published: March 12, 2014
consecutive piperolic acid residues, Ruzzo and co-workers observed unexpected amide bond hydrolysis and provided experimental evidence supporting the intermediacy of an oxazolinium ion. This hydrolytic sensitivity of peptidyl piperolic acid residues was exploited by Zajdel, Subra, and co-workers in the design of piperolic resin linkers for solid-phase applications.

The closest precedent for the observations reported in this work comes from Goodman and co-workers, who observed amide hydrolysis under mild conditions in a series of N-benzoyl-N-methylAlb-Phe-OMe analogues (4; Scheme 2). It was demonstrated that this instability is a function of the remote benzoyl groups on the N-terminal amino acid of this unusual dipeptide and that the rate of hydrolysis was correlated with the Hammett $\sigma$ constants of the benzoyl substituents, G. Analysis of the data established the existence of a linear free-energy relationship and supported a mechanism involving the intermediacy of an oxazolinium ion that proceeded from the tertiary amide.

To our knowledge, there are no reports of mild hydrolyses of the simple N-acylated primary amides or peptides represented by 1. We were surprised, then, in our earlier published work synthesizing 1 (Scheme 3, $R^3 = H$) from the benzophenone imine of Rink MBHA glycine amide 7 to observe small amounts of amide bond hydrolysis products 3 under TFA cleavage conditions. This occurred when $R^2$ was naphthyl but not when it was an Fmoc group.

Our focus in that report was to develop chemistry to incorporate unnatural side chains, $R^1$, into the $\alpha$-position of glycine with the acyl group, $R^2$, being used only to enable isolation and quantification of 1 ($R^3 = H$) produced in the key alkylation step. Therefore, $R^2$ was limited to the Fmoc group for all subsequent work quantifying the conditions and success of the alkylation chemistry, and the hydrolysis of the primary amide in the naphthyl case was mentioned only parenthetically.

However, when recently seeking to develop Scheme 3 further into a robust Distributed Drug Discovery (D3) lab that would permit the synthesis of large numbers of new compounds through widespread variation of both $R^1$ and $R^2$, it became apparent that the amide hydrolysis observed earlier was not an anomaly but a clear function of the nature of the $R^2$ group and that it would be important to understand the factors involved in this $R^2$-dependent hydrolytic instability so that its occurrence could be anticipated, predicted, and minimized. This report describes the achievement of that goal.

## RESULTS AND DISCUSSION

Creating D3 laboratories involves developing robust, detailed procedures that enable students, in the course of their regular undergraduate organic chemistry lab, to reproducibly synthesize many new biomimetic molecules as potential drug leads for neglected diseases. These procedures are often adapted from published chemistry. We were developing a new D3 laboratory, The Combinatorial Synthesis of N-Acylated Unnatural Amino Acid Amides, based on the published reaction sequence shown in Scheme 3. It would be compatible with a wide variety of $R^2$ groups obtained from carboxylic acids $R^2CO_2H$. For this adaptation to be successful, it would soon become apparent that understanding and addressing our earlier, parenthetical observation of $R^2$-dependent hydrolytic instability would be critically important.

This became clear when, in the course of this adaptation, two students conducted independent replicated syntheses of six new molecules, 1a–f (Scheme 3 and Table 1). The molecules were made through combinatorial reactions carried out in a $2 \times 3$ combinatorial grid that utilized two different carboxylic acids, $R^2CO_2H$, in rows A and B and three different alkylation agents, $R^1X$, in columns 1–3. The replicated and combinatorial nature of this study provided a concise set of compelling observations, revealing an $R^2$-dependent primary amide instability. If any one of the results for row A (A1–A3) of this grid had been obtained through a single, isolated experiment, then the presence of acid 3 might have been viewed as an anomaly. The fact that every reaction in row A gave significant amounts of carboxylic acids 3a–c as side products, that none of the reaction mixtures in row B contained hydrolyzed material, and that these results were independently repeated by two researchers suggested the presence of a systematic effect worthy of further inquiry. In addition, the combinatorial nature of these six experiments made it possible to narrow the important variable quickly to the acylating group $R^2$ (row A vs row B rather than columns 1–3) and to narrow further the common determining variable to the nature of the para substituent on the aromatic acylating agent.
On the basis of these observations, it was hypothesized that hydrolysis was a function of the electronic properties of the remote aromatic acyl group, R2, and that electron-donating groups would facilitate the hydrolysis, whereas electron-withdrawing substituents would suppress it. Accordingly, follow-up experiments were designed to explore this assumption systematically.

Predictive Nature of R2 Hammett σ Values on Hydrolytic Instability. To undertake a systematic investigation of the R2-dependent hydrolytic instability, compounds 10a–g, featuring a representative range of Hammett σ constants at the para position of the aromatic benzoyl group, were prepared on Rink Amide MBHA resins (2a–g), cleaved, and studied in solution (Scheme 4). After subjection to the original cleavage cocktail (95:5 TFA/water, 0.5 h, rt), all samples were filtered from resin so that subsequent time points (2, 4, 8, and 24 h) would reflect the instability of 10 solely in solution.

The study was designed to follow the initial formation of hydrolysis products 11a–g (either directly from 2a–g or through 10a–g during the 0.5 h cleavage) along with continuing formation of 11a–g from 10a–g in solution at increasing times postcleavage and postfiltration (2, 4, 8, and 24 h). The results from all time points are shown in Table 2 and are reported as the percent of acid 11 present. That the amount of acid increases with time of exposure to the cleavage cocktail after cleavage and filtration from the resin (all time points after 0.5 h) indicates that hydrolysis proceeds primarily in solution and comes directly from 10.

The data shows that electron-withdrawing substituents (10a–d, high σ values) suppress hydrolysis, whereas electron-donating substituents with the lowest Hammett σ values (10e–g) are associated with the most rapid hydrolyses to acids. This is readily apparent at all time points and mirrors the results obtained from the acylated N-methylAib-Phe-OMe dipeptides studied by Goodman and co-workers (Scheme 2). Plotting the percent acid present versus the Hammett σ constant for the data obtained at 24 h exposure revealed a linear relationship, which allowed qualitative predictions for other substituents to be made (see the Supporting Information).

This study was expanded (Table 3) to include various alkyl groups represented by R2. Resin precursor 12 was prepared and cleaved to 13, and time-dependent hydrolysis was studied. The results support those of our initial study: electron donation from R2 enhances production of N-acylated phenylalanines 14.

Optimization of Cleavage Conditions to Minimize Hydrolysis. Having demonstrated that R2-dependent amide hydrolysis can occur under typical resin-cleavage conditions, various cleavage methods were investigated to identify conditions that would maximize compound yield while minimizing subsequent hydrolysis. In the earlier study of the Hammett constant-dependent hydrolysis of 10 to 11 (Table 2),
we had already determined that if solutions of 10 were immediately stored at −20 °C after the 0.5 h cleavage time at room temperature then subsequent hydrolysis to 11 was completely suppressed (see the Supporting Information). However, for a robust and uncomplicated D3 procedure, it was necessary to develop optimized cleavage methods that would not require the use of low temperatures. The cleavage of 12k to 13k and hydrolysis to 14k (Table 4) was used as the test case in evaluating a series of cleavage methods. Resin 12k was prepared and subjected to the varied cleavage conditions. The percent yield of 13k was determined in each case by quantitative liquid chromatography calibrated with authentic 13k (QLC, expressed as the percent yield of 13k), and the ratio of 13k to hydrolysis product 14k was determined by proton NMR. Included in the list of methods are mixtures composed of lower percentages of TFA, shorter reaction times, and mixtures in which water was replaced by triethylsilane as a carbocation scavenger.22 Hydrolysis was substantially reduced (higher 13k:14k ratios) by using cleavage cocktails consisting of 65 or 35% TFA or by elimination of water from the cocktail (entries 3 and 4). However, reducing the percentage of TFA (method E, entries 9 and 10) or reducing the time of cleavage (30 min vs 1 or 2 h) runs the risk of incomplete cleavage from the resin.

The data indicates that reducing cleavage time to 30 min results, without exception, in lower yields of 13k. The use of 35:60:5 TFA/DCM/water (method D, entries 7 and 8) resulted in the lowest yields, but, interestingly, when triethylsilane was substituted for water (method E, entries 9 and 10), this composition performed significantly better. The improved yields associated with triethylsilane may reflect its ability to irreversibly scavenge the benzhydryl carbocation derived from the resin.22 Scavenging by water to give the diarylcarbinol would be reversible under the TFA conditions, potentially resulting in reattachment of the cleaved product to the resin.

Table 2. Percent of Acid 11 from 10 at Various Exposure Times to 95:5 TFA/H2O

<table>
<thead>
<tr>
<th>starting material</th>
<th>X</th>
<th>σ</th>
<th>0.5 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>CN</td>
<td>0.66</td>
<td>0</td>
<td>trace</td>
<td>trace</td>
<td>&lt;1</td>
<td>1</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10b</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>trace</td>
<td>trace</td>
<td>1</td>
<td>3</td>
<td>495</td>
<td></td>
</tr>
<tr>
<td>10c</td>
<td>CF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.54</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>247</td>
</tr>
<tr>
<td>10d</td>
<td>Cl</td>
<td>0.23</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>13</td>
<td>33</td>
<td>42</td>
</tr>
<tr>
<td>10e</td>
<td>H</td>
<td>0.00</td>
<td>6</td>
<td>12</td>
<td>18</td>
<td>31</td>
<td>66</td>
<td>16</td>
</tr>
<tr>
<td>10f</td>
<td>Me</td>
<td>−0.17</td>
<td>12</td>
<td>26</td>
<td>35</td>
<td>54</td>
<td>90</td>
<td>7.5</td>
</tr>
<tr>
<td>10g</td>
<td>OMe</td>
<td>−0.27</td>
<td>19</td>
<td>43</td>
<td>58</td>
<td>80</td>
<td>99</td>
<td>3.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Linear regression analysis assigns a rho value, ρ, of −2.3 to this reaction; see the Supporting Information. <sup>b</sup>Resin still present at this time point. <sup>c</sup>See the Supporting Information. <sup>d</sup>Not determined. <sup>e</sup>Value for NH3<sup>+.</sup>

Table 3. Percent of Acid 14 Formed from 13 at Various Exposure Times to 95:5 TFA/H2O

<table>
<thead>
<tr>
<th>compound 14</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>0.5 h&lt;sup&gt;a&lt;/sup&gt;</th>
<th>6 h</th>
<th>23 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>9-fluorenylmethyloxy</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>4-MeO-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0</td>
<td>trace</td>
<td>3</td>
</tr>
<tr>
<td>c</td>
<td>Me</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>d</td>
<td>Me&lt;sub&gt;2&lt;/sub&gt;CHCH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>e</td>
<td>c-propyl</td>
<td>trace</td>
<td>15</td>
<td>59</td>
</tr>
<tr>
<td>f</td>
<td>2-naphthyl</td>
<td>3</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>g</td>
<td>4-MeO-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;CH≡CH</td>
<td>8</td>
<td>54</td>
<td>80</td>
</tr>
<tr>
<td>h</td>
<td>Me&lt;sub&gt;2&lt;/sub&gt;C</td>
<td>10</td>
<td>40</td>
<td>84</td>
</tr>
<tr>
<td>i</td>
<td>1-adamantyl</td>
<td>16</td>
<td>59</td>
<td>92</td>
</tr>
<tr>
<td>j</td>
<td>4-MeO-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>10</td>
<td>53</td>
<td>96</td>
</tr>
</tbody>
</table>

<sup>a</sup>Resin still present at this time point.

Table 4. Optimization of Cleavage Conditions of 12k as a Function of the Cleavage Method and Time

<table>
<thead>
<tr>
<th>entry</th>
<th>method&lt;sup&gt;j&lt;/sup&gt;</th>
<th>time</th>
<th>percent yield 13k&lt;sup&gt;b&lt;/sup&gt;</th>
<th>13k:14k&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>30 min</td>
<td>62</td>
<td>86:14</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>2 h</td>
<td>76</td>
<td>90:10</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>30 min</td>
<td>73</td>
<td>96:4</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>1 h</td>
<td>88</td>
<td>95:5</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>30 min</td>
<td>55</td>
<td>97:3</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>1 h</td>
<td>80</td>
<td>97:3</td>
</tr>
<tr>
<td>7</td>
<td>D</td>
<td>30 min</td>
<td>19</td>
<td>96:4</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>1 h</td>
<td>32</td>
<td>96:4</td>
</tr>
<tr>
<td>9</td>
<td>E</td>
<td>30 min</td>
<td>55</td>
<td>99:1</td>
</tr>
<tr>
<td>10</td>
<td>E</td>
<td>1 h</td>
<td>73</td>
<td>98:2</td>
</tr>
</tbody>
</table>

<sup>j</sup>Methods: (A) 95:5 TFA/H<sub>2</sub>O; (B) 95:5 TFA/Et<sub>3</sub>SiH; (C) 65:30:5 TFA/CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>SiH; (D) 35:60:5 TFA/CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O; and (E) 35:60:5 TFA/CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>SiH. <sup>b</sup>Measured by QLC. <sup>c</sup>Measured by 1H NMR.
the resin via the primary amide or by way of Friedel−Crafts alkylation of the aromatic ring. The combination of a 1 h cleavage, replacement of water with triethylsilane, and use of a lower percentage of TFA represents the preferred methods that are described by C and E (entries 6 and 10).

**R2-Dependent N-Acylated Peptide Instability.** Our observation and analysis of the mild hydrolysis promoted by remote aromatic acyl groups R2 on the N-terminus of simple N-acylated amino acid amides \( R^3 = H \) prompted an additional experiment documenting its implications for N-acylated peptide instability. Rink Amide MBHA and Wang N-acylated dipeptide resins 15 and 16, respectively, were prepared and subjected to cleavage (95:5 TFA/water, 0.5 h, rt, Scheme 5). Analysis by LC/MS was performed after 0.5, 6, and 24 h (Table 5).

### Table 5. Ratio of Dipeptide 17 or 18 to Hydrolysis Product 14j at Various Times

<table>
<thead>
<tr>
<th>source of 14j</th>
<th>0.5 h*</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>17:14j (from Rink resin)</td>
<td>∼99:1</td>
<td>89:11</td>
<td>60:40</td>
</tr>
<tr>
<td>18:14j (from Wang resin)</td>
<td>∼99:1</td>
<td>88:12</td>
<td>49:51</td>
</tr>
</tbody>
</table>

*Resin still present at this time point.

Although negligible hydrolysis occurred in the initial 30 min exposure, by 6 h, 10−15% of the resin-free mixture was composed of hydrolysis product 14j, increasing to nearly 50% by 24 h.

**Mechanistic Considerations.** Observations from the above studies are consistent with hydrolysis proceeding through a rate-limiting step in which a positively charged transition state is stabilized by an electron-donating R2 group. A proposed mechanism is depicted in Scheme 6. Initial protonation at the C-terminal amide carbonyl to give 19 is followed by cyclization to the tetrahedral intermediate 20. Collapse of the tetrahedral intermediate affords an oxazolinium ion (21) of the type originally proposed by Urban et al. and subsequently put forward by others. Hammett substituent effects arising from R2 (alkyl or aryl) might be expected to be operative in the cyclization of 19 to 20 or in the acid-catalyzed ring opening of 21 to 3.

**Possible Utility of N-Acyl Group-Dependent Lability.** In the future, the substituent-dependent amide hydrolytic lability we have observed could be advantageously used. With the appropriate choice of the aryl group, a peptide temporarily acylated with ArCO-AA-OH (Ar = electron-rich aryl group, AA = amino acid residue) could be converted back to the unacylated parent peptide under mild, perhaps even physiological, conditions. For example, if the peptide’s physical properties require modification to improve its pharmacological profile, then a highly electron-rich Ar group might permit ArCO-AA-OH to be temporarily attached and then removed in a prodrug strategy.
CONCLUSIONS

This report shows that amide bonds in simple N-acylated amino acid amide or peptide derivatives can be surprisingly unstable. When an electron-rich aromatic carboxylic acid is acylated to the amino nitrogen of a simple amino acid carboxamide or the N-terminus of a peptide, room-temperature trifluoroacetic acid/water mixtures result in hydrolysis of the amide bond four bonds away from the NH-acyl group. Even simple aromatic acyl derivatives, when appropriately substituted with electron-donating substituents, can promote facile remote amide cleavage. In particular, this hydrolysis has been found to be sensitive to the substituent effects of R² located on a remote N-terminal aromatic acyl group. In the case of N-benzoyl amides, the relative rate of hydrolysis can be predicted from the substituent’s Hammett σ value. After 24 h at room temperature, compounds having benzoyl acyl substituents with negative σ values are almost completely hydrolyzed, those with values between 0 and 0.60 are moderately hydrolyzed, and those with values ≥0.60 show minimal amide cleavage. Acyl groups derived from secondary or tertiary amines are electron-rich and also promote substantial hydrolysis after 6 h at room temperature.

This acyl group-dependent acid-catalyzed lability occurs under acidic conditions commonly employed when cleaving products from solid-phase resins. When these products are N-acylated peptides, the hydrolysis reaction could become problematic. To address this issue, a systematic evaluation of cleavage study reported in Table 1. A 25 mL SPPS vessel was charged with 1.364 mmol (0.50 mL, 0.50 mmol, 10 equiv) of Fmoc-Gly-OMe-Rink MBHA resin. The resin was washed with 3 × 15 mL of NMP and was then treated with 5 × 10 mL washes of 20% (v/v) piperidine in NMP delivered over a 35 min period. The protected resin was then washed with 3 × 15 mL of NMP and was treated with 1.09 g (6.01 mmol, 10.0 equiv) of benzenophene imine in 9 mL of NMP followed by 0.313 g (5.22 mmol, 8.72 equiv) of acetic acid in 1 mL of NMP. The vessel was rocked on an orbital shaker for 22 h and then drained, and the resin was washed with 3 × 10 mL each of NMP, THF, 3:1 THF/H₂O, THF, and DCM to give resin |ow rate of 1.0 mL/min. A linear gradient from 20% 1.1 MeCN/MeOH (5 mM NH₂OAc) and 80% water (5 mM NH₂OAc) to 100% 1:1 MeCN/MeOH (5 mM NH₂OAc) over 10 min was used. Detection was performed at 254, 214, and 210 nm. Percent amide and/or percent acid were calculated from the reported peak areas observed at 254 nm unless otherwise noted.

General Method. All reagents were commercially available and were used without further purification. Fmoc-protected amino acid-bound Rink Amide MBHA and Wang resins were purchased from Peptides International (Louisville, KY). Boc-protected amino acid-bound Merrifield resins were purchased from Novabiochem (EMD Millipore) or Polymer Laboratories (now Agilent). Combinatorial and parallel synthetic sequences and resin cleavage/hydrolysis experiments were performed in custom-made 3.5 mL glass reaction vessels containing a sintered glass frit and screw-cap ends fitted with poly(tetrafluoroethylene) (PTFE) septa all purchased from Chemglass (Vineland, NJ). The reaction vessels were secured in polypropylene Bill-Boards consisting of a 2 × 3 or 4 × 6 array of position holes fitted with rubber O-rings and were supplied by Leads Metal (Indianapolis, IN). Synthetic sequences for the preparation of advanced intermediate resins were performed in 25 or 50 mL solid-phase peptide synthesis vessels (SPPS) purchased from Chemglass. Rotation of Bill-Boards was accomplished using a spin rod fitted with three 6-brackett metal assemblies custom-made by Leads Metal and was driven by a Jen-Air rotisserie motor or using brackets to fit 2 × 3 or 4 × 6 Bill-Boards held in a rotovap assembly.
and the Bill-Boards were rotated for 20 min, the vessels were drained, and the resins were washed with 3 mL of THF, 2 × 2.5 mL of 0.2 M disopropylethylamine in NMP, and 2 × 2.5 mL of NMP to give resins 9 (Scheme 3, R1 = 4-methylbenzyl, ethyl, allyl). 4-Methylbenzoic acid and 4-trifluoromethylbenzoic acid (0.25 M, 1.0 mL, 0.25 mmol, 5.0 equiv) in 0.25 M 1-hydroxybenzotriazole (HOBr) in NMP were added, respectively, to row A vessels A1–A3 and row B vessels B1–B3. Each vessel was then treated with 0.50 mL (0.25 mmol, 5.0 equiv) of 0.50 M disopropylcarbodiimide in NMP, and the Bill-Boards were rotated for 22 h. The vessels were drained, and the resins were washed with 2 × 3 mL each of NMP, THF, and 3 × 3 mL of DCM to afford resins 2 (Scheme 3). Cleavage of each resin with 95:5 TFA/water for 2 h was performed as described above (General Procedure of Resin Cleavage).

LC/MS and 1H NMR Results of TFA-Mediated Cleavage of 2 × 3 Combinatorial Bill-Boards (Scheme 3 and Table 1). Cleavage of 2 gave the crude products in Table 1 (see General Procedure of Resin Cleavage). Analytical data for crude products 1 (Z = NH2) and 3 (Z = OH) include LC/MS and diagnostic peaks in the 1H NMR.

α-[4-(Methylbenzyl)amino]-4-methylbenzenepropanamide (1a) and N-(4-Methylbenzyl)amino-4-methylphenylalanine (3a). For 1a: 1H NMR (500.13 MHz, CDCl3): δ 2.30 (s), 2.38 (s), 3.19 (dd), 3.29 (dd), 3.61 (dd), 5.21 (s), 5.75 (d); LC/MS (ESI): 870 min, m/z 297 [M + H]+. For 3a: 1H NMR (500.13 MHz, CDCl3): δ 3.13 (2dd), 4.94 (dd), 7.59 (d); LC/MS (ESI): 6.36 min, m/z 298 [M + H]+.

Preparation of Resin 10a–g (R1 = 4-Methylbenzyl). The array of 35 vessels each containing 50.0 μmol of resin 9 was treated in the following manner: 1.0 mL of a 0.25 M solution of row 1 carboxylic acid 1RCOOH (250 μmol, 5.0 equiv) dissolved in 0.25 M 1-hydroxybenzotriazole (250 μmol, 5.0 equiv, HOBr) in NMP was added to each of the five vessels in row 1 followed by 0.5 mL of a 0.50 M solution of disopropylcarbodiimide (250 μmol, 5.0 equiv, DIC) in NMP. The five vessels in each of the remaining six rows were treated in an identical manner with its assigned carboxylic acid 1RCOOH (250 μmol, 5.0 equiv, DIC) and HOBt. The Bill-Board was rotated for 2 days, and the vessels were drained and then washed with 3 × 1.5 mL each of NMP, THF, and DCM to give resins 2a–g.

TFA-Mediated Cleavage of Resins 2a–g to 10a–g and 11a–g (Scheme 4, Hambett Study, Table 2). Each of the 35 vessels in the 5 × 7 array (50 μmol each) was treated with 1.5 mL of 95:5 TFA/H2O and was rotated at room temperature for 30 min. All vessels were then drained into collection vials, and the resins were washed with 1 mL of 95:5 TFA/H2O. A 90 μL aliquot from the combined filtrates of each acylated amide 10a–g in column 1 (representing the 0.5 h exposure) was removed and immediately evaporated to dryness under a stream of nitrogen. These samples were diluted with acetonitrile and analyzed by LC/MS. The remainder of each filtrate in column 1 (0.5 h exposure) was then stored at −20 °C and assayed by LC/MS again after 48 h. These 1 filtrates, stored at −20 °C, would be worked up for the purpose of isolating primary amides 10a–g. Filtrates in columns 2–5 were allowed to stand at room temperature for 1.5, 3.5, 7.5, and 23.5 h, respectively, and were then assayed by LC/MS (Table 2). After sampling for analysis, filtrates were stored at −20 °C. Complete conversion of amides to acids 10a–g to 11a–g (S = H, Me, and OMe), for the purpose of isolation and characterization, was accomplished by allowing their column 5 filtrates to stand at room temperature for 5 days.

Isolation of Amides 10a–g. After storage at −20 °C for 2 days, each solution from column 1 was added to 100 mL of cold 1.0 N sodium hydroxide. The mixture was then extracted with two 20 mL portions of DCM. The combined extracts were dried over Na2SO4 and then washed with 3 mL each of 20% (v/v) argon for 45 min and was then stored overnight.

Preparation of Resin 9 (R1 = 4-Methylbenzyl) and Distribution to 3.5 mL Reaction Vessels. Resin 9 was treated with 30 mL of 2.1 TFA/1.0 N HCl, and the vessel was rocked for 40 min and then drained. The resin was washed with 3 × 20 mL of THF, neutralized with 3 × 20 mL washes of 0.2 M disopropylethylamine (DIEA) in NMP, and then washed with 3 × 20 mL of NMP to give resin 9. Resin 9 was then quantitatively transferred to a 400 mL beaker using 100 mL of NMP to facilitate the transfer. Dichloromethane (50 mL) was then added, and the composition was adjusted to achieve neutral buoyancy. A final volume of 200–250 mL was then equally dispensed 2 mL at a time into a 5 column × 7 row array of reaction vessels with uncapped bottoms held in two 4 × 6 Bill-Boards (see General Procedure for the Preparation and Distribution of Isopycnic Suspensions of Resins). Each vessel was then washed with 3 × 2 mL of NMP and then capped.
acetone to give 6.9 mg (51%) amides (Scheme 4).

\[ \text{Scheme 4} \]

\[ \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ -\text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{The Journal of Organic Chemistry} \]

\[ \alpha \text{-[4-Chlorobenzyl]amino]-4-methylbenzepropanamide (10c). Yield 4.1 mg (23%), mp 220–224 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \alpha \text{-[4-Methylbenzyl]amino]-4-methylbenzepropanamide (10f). Yield 8.0 mg (54%), mp 206–210 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \alpha \text{-[4-Methoxybenzyl]amino]-4-methylbenzepropanamide (10g). Yield 6.6 mg (42%), mp 210–212 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \alpha\beta\text{-acetylenic ester (3 mol/L) to the TFA solution, the vials containing the bulk of the} \]

\[ \text{B, C; 1H NMR (500.13 MHz, DMSO-d6):} \]

\[ \alpha \text{-[4-Methoxybenzyl]amino]-4-methylbenzepropanamide (10g). Yield 6.6 mg (42%), mp 210–212 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{Preparation of} \text{-Acetylated (5)-Phenylalanine-NH-Rink Amide MBHA Resins 12b–j. A 50 mL SPPS vessel was charged with 780–940 mg (250–300 μmol) of Fmoc-Phe-NHL-AMide MBHA resin (0.32 mmol/g, Peptides International) and treated with 20% of} \]

\[ \text{and eluting the desired material with 7:3 hexanes/acetonitrile to give 5.3 mg (36%) 11g as an amorphous solid, mp} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{B, C; 1H NMR (500.13 MHz, DMSO-d6):} \]

\[ \alpha \text{-[4-Methoxybenzyl]amino]-4-methylbenzepropanamide (10g). Yield 6.6 mg (42%), mp 210–212 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{Preparation of} \text{-Acetylated (5)-Phenylalanine-NH-Rink Amide MBHA Resins 12b–j. A 50 mL SPPS vessel was charged with 780–940 mg (250–300 μmol) of Fmoc-Phe-NHL-AMide MBHA resin (0.32 mmol/g, Peptides International) and treated with 20% of} \]

\[ \text{and eluting the desired material with 7:3 hexanes/acetonitrile to give 5.3 mg (36%) 11g as an amorphous solid, mp} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{B, C; 1H NMR (500.13 MHz, DMSO-d6):} \]

\[ \alpha \text{-[4-Methoxybenzyl]amino]-4-methylbenzepropanamide (10g). Yield 6.6 mg (42%), mp 210–212 °C; H NMR (500.13 MHz, DMSO-d6):} \]

\[ J_{\text{H}} = 8.0 \text{ Hz, 2H), 7.49 (br s, 1H), 7.79 (d, J = 8.9 \text{ Hz, 2H), 8.29 (d, J = 8.4 \text{ Hz, 1H;}} \]

\[ \text{C NMR (125.77 MHz, DMSO-d6):} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{Preparation of} \text{-Acetylated (5)-Phenylalanine-NH-Rink Amide MBHA Resins 12b–j. A 50 mL SPPS vessel was charged with 780–940 mg (250–300 μmol) of Fmoc-Phe-NHL-AMide MBHA resin (0.32 mmol/g, Peptides International) and treated with 20% of} \]

\[ \text{and eluting the desired material with 7:3 hexanes/acetonitrile to give 5.3 mg (36%) 11g as an amorphous solid, mp} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{Preparation of} \text{-Acetylated (5)-Phenylalanine-NH-Rink Amide MBHA Resins 12b–j. A 50 mL SPPS vessel was charged with 780–940 mg (250–300 μmol) of Fmoc-Phe-NHL-AMide MBHA resin (0.32 mmol/g, Peptides International) and treated with 20% of} \]

\[ \text{and eluting the desired material with 7:3 hexanes/acetonitrile to give 5.3 mg (36%) 11g as an amorphous solid, mp} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]

\[ \text{Preparation of} \text{-Acetylated (5)-Phenylalanine-NH-Rink Amide MBHA Resins 12b–j. A 50 mL SPPS vessel was charged with 780–940 mg (250–300 μmol) of Fmoc-Phe-NHL-AMide MBHA resin (0.32 mmol/g, Peptides International) and treated with 20% of} \]

\[ \text{and eluting the desired material with 7:3 hexanes/acetonitrile to give 5.3 mg (36%) 11g as an amorphous solid, mp} \]

\[ \text{d} \delta \text{H, 2H);}13\text{C NMR (125.77 MHz, DMSO-} \]
The Journal of Organic Chemistry

9H-Fluoren-9-ylmethyl N-[2-Amino-2-oxo-1-(phenylmethyl)ethyl]carbamate [Fmoc-Phe-NH2] (13a). The filtrate was evaporated to dryness under a stream of nitrogen to give 6.6 mg of crude material. Trtitration under diethyl ether afforded 6.1 mg (33%) of 13a as an amorphous solid, mp 192–194 °C (dec); 1H NMR (500.13 MHz, CDCl3) δ 0.27 (dd, J = 13.9 and 4.7 Hz, 1H), 2.98 (dd, J = 13.7 and 4.7 Hz, 1H), 3.22 (dd, J = 14.0 Hz, 1H), 3.34 (dd, J = 13.9 Hz, 1H), 3.71 (s, 3H), 4.43 (dd, J = 9.3, 8.5, and 4.7 Hz, 1H), 6.77 (d, J = 7.2 Hz, 2H), 7.08 (d, J = 7.5 Hz, 2H); 13C NMR (125.77 MHz, CDCl3) δ 37.4, 45.8, 53.1, 60.4, 73.1, 124.1, 126.1, 127.6, 128.0, 128.2, 129.3, 129.5, 133.6, 136.8, 137.4, 138.5, 148.3, 151.5, 172.7, 173.8; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C24H22N2O3Na, 409.1308; found, 409.1305.

4-Methoxy-α-(2-phenylacetyl)aminobenzene propanamide (13d). The filtrate was added carefully to 25 mL of cold 2 M NaOH, and the mixture was extracted with 2 × 20 mL of DCM. The combined extracts were dried (Na2SO4) and concentrated to give 7.7 mg of crude material from which 2.0 mg of pure 13d was obtained by reverse-phase HPLC on a Dynamax Microsorb C-18, 5 μm column (21.4 × 250 nm) eluting isocratically with 60:40 1:1 MeCN/MeOH (with 5 mM ammonium acetate)/water (with 5 mM ammonium acetate) at 5 mL/min and detection at 254 nm to afford 1.9 mg (30%) of 13d (15S:15R = 3:1 mixture of E/Z isomers); confirmed by 1H and 13C NMR.

In a 25 mL round-bottom flask, 1H NMR (500.13 MHz, CDCl3) δ 1.12 (s, 9H), 3.09 (m, 1H), 4.69 (dd, J = 14.4 and 7.2 Hz, 1H), 5.59 (br s, 1H), 6.12 (br s, 1H), 6.30 (br d, J = 7.2 Hz, 1H), 7.22–7.25 (m, 2H), 7.28–7.31 (m, 2H); 13C NMR (125.77 MHz, CDCl3) δ 27.3, 38.0, 38.7, 53.8, 127.1, 128.7, 129.3, 136.6, 173.4, 178.7; HRMS (ESI-TOF) m/z: [M + H]+ calcd for C22H21NO4, 325.1547; found, 325.1552.

α-[(2,2-Dimethyl-1-oxoaryl)amino]benzene propanamide (13h). 

α-[(3-Methyl-1-oxobutyl)amino]benzene propanamide (13e). 

α-[(Cyclopropylcarbonyl)amino]benzene propanamide (13f).

α-[(Cyclopropylcarbonyl)carbonyl]amino]benzene propanamide (13g).


α-[(4-Methoxybenzoyl)amino]benzene propanamide (13j) and N-[(4-Methoxybenzoyl)phenylalamine (14j). In a 25 mL solid-phase peptide synthesis (SPPS) vessel, Fmoc-Phe-NH-Rink Amide MBHA resin (576 mg, 190 μmol, 0.33 mmol/g, Peptides International) was deprotected using 30% piperidine in NMP. The amino resin was then treated with 3.77 mL of a 0.25 M solution of 4-methoxybenzoic acid (143 mg, 942 μmol, 4.96 equiv) in 0.25 M N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-y)uronium hexafluorophosphate (HBTU) (361 mg, 952 μmol, 5.01 equiv) in NMP followed by 3.77 mL of a 0.50 M solution of disopropylethylamine
The Journal of Organic Chemistry

Article

(1880 μmol, 9.91 equiv) in NMP. The vessel was rocked at room temperature on an orbital shaker for 22 h and was then drained. The resin gave a negative Kaiser test and was then washed with 4 × 10 mL of 1 M NaOH and once with 10 mL of water and was then dried (Na₂SO₄). Concentration gave 19.6 mg (35%) of 13k as an amorphous solid, mp 201—204 °C. ¹H NMR (500.13 MHz, DMSO-d₆): δ 2.97 (dd, J = 13.6 and 10.7 Hz, 1H), 3.10 (dd, J = 13.6 and 4.0 Hz, 1H), 3.79 (s, 3H), 4.83 (dd, J = 9.4 and 4.9 Hz, 1H), 6.94 (d, J = 8.9 Hz, 2H), 7.19 (m, 1H), 7.24—7.28 (m, 4H), 7.70 (d, J = 8.9 Hz, 2H); ¹³C NMR (125.77 MHz, CDCl₃): δ 31.11 (d, J = 13.9 and 9.4 Hz, 1H), 3.32 (d, J = 5.0 Hz, 1H), 3.83 (s, 3H), 4.83 (dd, J = 9.4 and 4.9 Hz, 1H), 6.94 (d, J = 8.9 Hz, 2H), 7.19 (m, 1H), 7.24—7.28 (m, 4H), 7.70 (d, J = 8.9 Hz, 2H); ¹⁵N NMR (125.77 MHz, CDCl₃): δ 38.2, 55.6, 55.9, 114.7, 127.4, 127.8, 129.4, 130.3, 138.8, 164.1, 169.7, 175.0 (only 12 of 13 lines were observed); HRMS (ESI-TOF) m/z: [M + H⁺] calc for C₁₀H₈N₄O₄: 204.0873; found, 204.0873. LC/MS (ESI): 8.19 min, m/z 283 [M + H⁺].

Compound 13k was synthesized in solution phase using the method of Uchida et al. and was purified for use as the authentic standard in the quantitative LC measurements (optimization of cleavage conditions, Table 4). A 25 mL SPPS vessel was charged with 1.857 g (0.5478 mmol, 0.295 mmol/g) of Fmoc-Phe-NH-Rink Amide MBHA resin. The resin was swelled by washing with 3 × 10 mL of NMP followed by 10 mL of 20% (v/v) piperidine in NMP. The resin was then treated with 12 mL of 20% piperidine in NMP. The combined basic extracts were adjusted to pH 2 with a few milliliters of cold 1:1 acetone/water to afford 97 mg of crude 13k, which was triturated under hot methanol to give 29.5 mg (9%) of the QCL calibration sample. ¹H NMR (500.13 MHz, DMSO-d₆): δ 2.33 (s, 3H), 2.98 (dd, J = 13.6 and 10.7 Hz, 1H), 3.10 (dd, J = 13.6 and 4.0 Hz, 1H), 4.62 (dd, J = 10.7, 8.6, and 4.2 Hz, 1H), 7.09 (br s, 1H), 7.15 (t, J = 7.3 Hz, 1H), 7.24 (m, 4H), 7.32 (d, J = 7.5 Hz, 2H), 7.52 (t, J = 7.0 Hz, 2H), 7.70 (d, J = 8.2 Hz, 2H); ¹³C NMR (125.77 MHz, CDCl₃): δ 20.8, 37.1, 54.4, 60, 127.3, 127.9, 128.6, 129.0, 131.2, 138.5, 141.0, 165.3, 175.3. LC/MS (ESI): 8.19 min, m/z 283 [M + H⁺].

For storage for 3 days.

The crude material (9.3 mg) was purified by cyanosilica gel chromatography (500 mg prepacked column, eluting with 1:1 and then 3:1 hexanes/DMSO) to give 29.5 mg of 13k as an amorphous solid, mp 229—232 °C; ¹H NMR (500.13 MHz, CDCl₃): δ 3.11 (d, J = 13.9 and 9.4 Hz, 1H), 3.32 (d, J = 5.0 Hz, 1H), 3.83 (s, 3H), 4.83 (dd, J = 9.4 and 4.9 Hz, 1H), 6.94 (d, J = 8.9 Hz, 2H), 7.19 (m, 1H), 7.24—7.28 (m, 4H), 7.70 (d, J = 8.9 Hz, 2H); ¹³C NMR (125.77 MHz, CDCl₃): δ 38.2, 55.6, 55.9, 114.7, 127.4, 127.8, 129.4, 130.3, 138.8, 164.1, 169.7, 175.0 (only 12 of 13 lines were observed); HRMS (ESI-TOF) m/z: [M + H⁺] calc for C₁₀H₈N₄O₄: 204.0873; found, 204.0873. LC/MS (ESI): 8.19 min, m/z 283 [M + H⁺].

N-(4-Methoxybenzoyl)-Phe-Ala-NH-Rink Amide MBHA Resin (15) and N-(4-Methoxybenzoyl)-Phe-Ala-O Wang Resin (16, Scheme 5).

For storage for 3 days.

For storage for 3 days.

Scheme 5).

For storage for 3 days.

For storage for 3 days.

For storage for 3 days.

Table 4)

For storage for 3 days.

Table 4).

For storage for 3 days.

Table 4).

For storage for 3 days.

Table 4).

For storage for 3 days.

Table 4).

For storage for 3 days.

Table 4).

For storage for 3 days.
173.6, 177.3; HRMS (ESI-TOF) m/z: [M + H]+ calcd for C20H23N2O5 371.1601; found 371.1604.

■ ASSOCIATED CONTENT

Supporting Information

1H and 13C NMR data and spectra and graphical treatments of the Hammett and cleavage method studies. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors
*E-mail: modonnel@iupui.edu (M.J.O.).
*E-mail: wscott@iupui.edu (W.L.S.).

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the National Institutes of Health (RO1-GM28193), the National Science Foundation (NSF/DUE-1140602, NSF/MRI-CHE-0619254, and NSF/MRI-DBI-0821661), and the Indiana University Purdue University Indianapolis STEM Summer Scholars Institute for their generous support of this work.

■ REFERENCES


14. Although it was realized that the potential for partial racemization existed, no attempt was made to establish the stereochromaticity at the α-carbon of 14.


24. Although it was realized that the potential for partial racemization existed, no attempt was made to establish the stereochromaticity at the α-carbon of 14.


34. Steward, J. M.; Young, J. D. In Solid-Phase Peptide Synthesis, 2nd ed.; Pierce Chemical Company: Rockford, IL, 1984: pp 38–44.

35. Although it was realized that the potential for partial racemization existed, no attempt was made to establish the stereochromaticity at the α-carbon of 14.


