# *p*-Cresol As a Reversible Acylium Ion Scavenger in Solid-Phase Peptide Synthesis

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**Abstract:** In this work we have defined the nature of the *p*-cresol and *p*-thiocresol adducts generated from acylium ions during HF cleavage, following contemporary Boc/benzyl solid-phase peptide synthesis. Contrary to the results in previous reports, we found that both *p*-cresol and *p*-thiocresol predominantly form aryl esters under typical cleavage conditions. Initially we investigated a number of small peptides containing either a single glutamate residue or a *C*-terminal long-chain amino acid which allowed us to unambiguously characterize the "scavenged" side products. Whereas, the *p*-cresol esters are stable at 0 °C they rearrange irreversibly at higher temperatures (5−20 °C) to form aryl ketones. By contrast, *p*-thiocresol esters do not undergo a Fries rearrangement but readily undergo further additions of *p*-thiocresol to form ketenebisthioacetals and trithio ortho esters, even at low temperatures. Importantly, we found by LC/MS and FT-ICR MS analysis that peptides containing *p*-cresol esters at glutamyl side chains are susceptible to amidation and fragmentation reactions at these sites during standard mild base workup procedures. The significance of these side reactions was further demonstrated in the synthesis of neutrophil immobilization factor, a 26-residue peptide, containing four glutamic acid residues. The side reactions were largely avoided by mild hydrogen peroxide-catalyzed hydrolysis which converted the *p*-cresol adducts to the free carboxylic acids in near quantitative yield. The choice of *p*-cresol as a reversible acylium ion scavenger when coupled with the simple workup conditions described is broadly applicable to Boc/benzyl peptide synthesis and will significantly enhance the quality of peptides produced.

## Introduction

One of the main challenges in peptide synthesis is to establish synthetic routes to homogeneous products of defined covalent structure. Since Merrifield introduced solid-phase peptide synthesis (SPPS),[1,2] new synthetic protocols have continually been formulated toward greater efficiency in peptide assembly and cleavage from the solid support. Despite this progress, side reactions still plague many syntheses, often resulting in low yields and poor homogeneity of the target peptide, especially for peptides over 20 residues.

In Boc SPPS[3a] the protection strategy adheres to the principle of graded acid lability. Following chain assembly, cleavage from the solid support and side chain deprotection is generally performed by treating the resin bound peptides with strong acids and scavengers. The most effective and commonly used acid for this purpose is anhydrous hydrogen fluoride,[3b] although acids such as hydrogen bromide[4,5] and trifluoromethanesulfonic acid in trifluoroacetic acid have also been used successfully.[6]

HF is a highly volatile, nonoxidizing acid with a strong protonating ability and forms an excellent solvating medium for peptides. When used in high concentrations, HF removes all the common protection groups efficiently via an $S_N1$ process, thereby generating carbocations, which are subsequently trapped by the added scavenger(s). Several HF-induced side reactions have been observed and thoroughly studied, for example: (1) side chain alkylation at tyrosine,[7] tryptophan,[8,9] methionine,[10] and cysteine[11,12] residues due to insufficient scavenging of various carbocations; (2) formation of succinimide at aspartyl residues, which, upon hydrolysis, ring open to form both α-(backbone) and *β*-(side chain) peptides;[13] (3) $N \rightarrow O$ acyl

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(3) (a) Boc SPPS refers to use of *tert*-butoxycarbonyl and benzyl temporary and permanent protecting groups, respectively. Abbreviations used are the following: Bzl, benzyl; $CH_3CN$, acetonitrile; COSY, correlation spectroscopy; Da, Dalton; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ES-MS, electrospray mass spectrometry; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; $H_2O_2$, hydrogen peroxide; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HF, anhydrous hydrogen fluoride; LC/MS, liquid chromatography mass spectrometry; Merrifield resin, chloromethylpolystyrene−divinylbenzene (200−400 mesh); MRP14, migration inhibitory factor related protein 14 kDa; NIF, neutrophil immobilization factor; NMR, nuclear magnetic resonance; Pam, phenylacetamidomethyl polystyrene resin; RP-HPLC, reverse-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid. Standard IUPAC single and triple letter codes for amino acids are used throughout. (b) Sakakibara, S.; Shimonishi, Y. *Bull. Chem. Soc. Jpn.* **1965**, *38*, 1412−1413.

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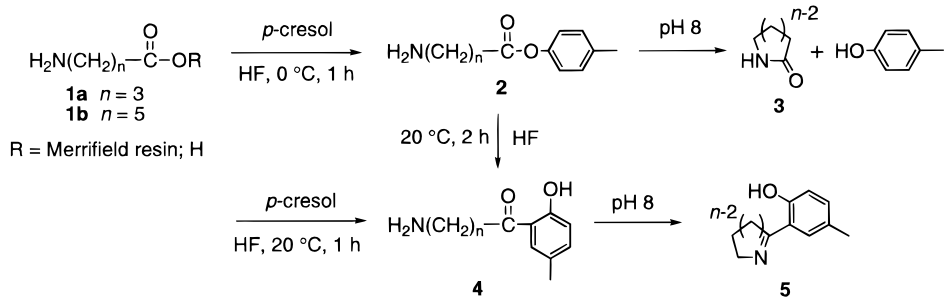
**Scheme 1.** Reactions of $\omega$-Amino Acid Derivatives in HF/*p*-Cresol (9:1) at 0 and 20 °C




transfer of serine- and threonine-containing peptides from the backbone nitrogen atom to the side chain oxygen atom on the serine and threonine residues[14−17] (reversed by mild base treatment); (4) acylation via acylium ion formation on glutamyl side chains[18−20] and on *C*-terminal $\omega$-amino acids.[21] The extent of these side reactions is largely controlled by the cleavage conditions, including time, temperature, and HF concentration, and by the protection groups used in the synthesis. In an effort to minimize cleavage side reactions, Tam et al.[22] proposed the use of a "low-HF" mixture (HF/dimethyl sulfide/*p*-cresol, 25:65:10, v/v) where initial cleavage of most side chain groups follows an $S_N2$ mechanism. This is then followed by a "high-HF" treatment to facilitate complete side chain deprotection and cleavage from the solid support (HF/*p*-cresol/*p*-thiocresol, 18:1:1, v/v). Since most of the carbocation precursors are removed under low-HF conditions, the number of carbocations produced during the "high-HF" step would be greatly reduced and, in principle, the number of side products would be greatly reduced as well.

However, even under these controlled conditions acylium ions may still be efficiently formed from the unprotected carboxylic acids.[21] If not scavenged, the $\gamma$-glutamyl acylium ion ring closes to the α-nitrogen atom in the backbone thus forming a pyrrolidinone which is then susceptible to several subsequent reactions during the workup of the cleavage mixture.[18] In the presence of aromatic scavengers, such as anisole, the acylium ion is trapped irreversibly through a Friedel−Crafts acylation generating an aryl ketone.[18,23] For long-chain carboxylic acids separated from the amide backbone by more then two methylenes, acylium ion formation under neat HF cleavage conditions is nearly quantitative and results in total loss of target peptide.[21] Effective Boc solid-phase synthesis of homogeneous peptides containing glutamyl residues and/or long-chain carboxylic acids would benefit greatly from a new scavenging mechanism. Such a mechanism should, on the one hand, rapidly trap the acylium ion, and on the other hand, the trapped product should be convertible to the target peptide via a mild chemical procedure that does not affect other functionalities in the molecule.[18]

*p*-Cresol and *p*-thiocresol are commonly used scavengers for HF cleavage of protected peptide resins.[24] *p*-Cresol is highly effective in preventing tyrosine ring benzylation and aspartimide formation during HF-mediated cleavage,[12,25] while *p*-thiocresol improves cation scavenging and increases deprotection rates of the protected forms of tryptophan, arginine or cysteine residues.[11,12,22,26] It is generally believed that these scavengers act in a fashion similar to anisole with acylium ion trapping leading to aryl ketones.[21,23] However, recent data from our laboratory led us to believe that the formation of aryl ketones with cresol-based scavengers did not occur under standard HF conditions (0 °C, 1 h).[27]

Here we report a detailed study on the nature of the *p*-cresol and *p*-thiocresol scavenging of acylium ions and examine their new role in peptide synthesis as reversible acylium ion scavengers for improving yields and homogeneity of target peptides.

## Results and Discussion

**HF Cleavage of $\omega$−Amino Acids.** Long-chain amino acids at the *C*-terminus of peptides nearly quantitatively generate acylium ions in anhydrous HF[21] under standard cleavage conditions. They thus provided an excellent model for our initial examination of the nature of *p*-cresol scavenging and its accompanying side reactions.

We subjected amino acid derivatives **1a** to standard HF cleavage conditions (0 °C, 1 h) in the presence of *p*-cresol as scavenger (Scheme 1). In both cases (R = Merrifield resin or H), the main component (> 85%) of the residue after HF evaporation was characterized unambiguously by ES-MS and $^1$H and $^{13}$C NMR as ester **2a**. That **2a** is indeed an ester and not the expected ketone is evident from the *para*-substitution pattern of the phenyl ring in the $^1$H NMR spectrum and the carbonyl resonance in the $^{13}$C NMR spectrum at 183 ppm. In 0.1 M $NH_4HCO_3$, ester **2a** was converted to lactam **3a**, as evidenced by ES-MS and RP-HPLC comparison with an authentic sample. Similarly, aminocaproic acid derivatives **1b** mainly (> 85%) generated ester **2b** under standard HF/*p*-cresol cleavage conditions.

By contrast, longer cleavage times and higher temperatures caused substantial formation of ketones **4** via a HF-catalyzed

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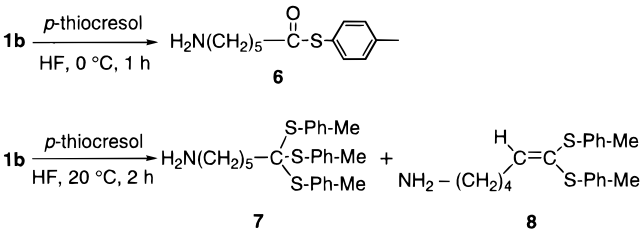
**Table 1.** HF/*p*-Cresol (9:1) Treatment of $\omega$-Amino Acids **1**, Effect of Cleavage Conditions on the Product Distribution

| *n* | (**1**) R | temp (°C) | time (h) | **2** (%) | **4** (%) | **5** (%) |
|---|---|---|---|---|---|---|
| 3 | resin | −5 | 1 | >85 | <1 | <1 |
| 5 | resin | −5 | 2 | 80 | 15 | <1 |
| 5 | H | 0 | 2 | 75 | 20 | 2 |
| 3 | H | 20 | 12 | <1 | <1 | 85 |
| 5 | H | 20 | 12 | <1 | 30 | 50 |

[a] Yields were estimated by integration of the $^1$H NMR spectrum of the crude cleavage product.

**Scheme 2.** Reactions of $\omega$-Amino Acid Derivatives in HF/*p*-Thiocresol (9:1)




Fries rearrangement[28] of the initially formed esters **2**. Ketone **4a** was not directly detected as it cyclized spontaneously and quantitatively to the Schiff's base **5a**. The correct molecular weight, $^1$H NMR, and a characteristic $^{13}$C NMR signal at 183 ppm confirmed the imine structure over that of the intermediate ketone. For **1b**, LC/MS and $^{13}$C NMR analyses of the crude residue (HF, 20 °C, 12 h) indicated the presence of both ketone **4b** and cyclic imine **5b** from resonances at 208 and 183 ppm, respectively. Cyclization of ketone **4b** to the Schiff's base **5b** was driven to completion by base treatment of the crude mixture. The effect of time and temperature on the product distribution in the crude residue based on $^1$H NMR analysis is given in Table 1. All *p*-cresol esters had rearranged to their corresponding aryl ketones after standing in HF for 12 h at 20 °C. Through careful control of the cleavage temperature and time, the Fries rearrangement of the *p*-cresol ester can be largely avoided and results in nearly full conversion of the acylium ion to the *p*-cresol ester. The product distribution was similar for both the resin-bound and free aminobutyric acid, which indicates that "deprotection" of the acid function prior to high-HF treatment (e.g., via a low−high HF treatment[22]) will not prevent acylium ion formation in the cleavage process.

Similar to the above, HF treatment of compounds **1b** at 0 °C in the presence of *p*-thiocresol produces thioesters **6** (> 85%) (Scheme 2). If a mixture of *p*-cresol and *p*-thiocresol (1:1) is used, as is often the case in HF cleavages of peptidyl resins, thioester **6** is still the predominant product, with only traces of the *O*-ester **2** present. The RP-HPLC profile (data not shown) of the crude cleavage mixtures indicated the presence of other side products (**7** and **8**) corresponding to multiple additions of *p*-thiocresol to the acylium ion derived from **1b**.

Aryl thioesters are not susceptible to Fries rearrangement[29] and thus will not generate aryl thioketones in anhydrous HF, even at higher temperatures or longer cleavage times. To verify this and further elucidate the nature of the multiple *p*-thiocresol adducts, aminocaproic acid on Merrifield resin (**1b**) was treated with HF/*p*-thiocresol (9:1) at 20 °C for 2 h. The major component of the reaction product (>85%) was characterized as the trithio ortho ester **7** ($M_r$ 467). A second component, **8** (5%), possessed a molecular weight corresponding to the loss of one molecule of *p*-thiocresol from **7** ($M_r$ 343). Trithio ortho esters that contain α protons have been reported to readily eliminate thio alcohol and generate ketenebisthioacetals.[30,31] Thus, we were able to convert the trithio ortho ester **7** to the ketenebisthioacetals (**8**) by refluxing an aqueous solution containing 10 equiv of $ZnCl_2$.[31] The $^1$H and $^{13}$C NMR data of the "double adduct" confirmed structure **8**. We also observed that the trithio ortho ester **7** readily eliminated one *p*-thiocresol molecule under standard ES-MS conditions with only a small $MH^+$ signal observed at 468 *m*/*z* with the predominant signal at 344 *m*/*z*. Thus, it was clear that long-chain amino acids and probably glutamyl side chains are susceptible to formation of trithio ortho esters and ketenebisthioacetals under standard HF/*p*-thiocresol cleavage conditions.

A proposed mechanism for the formation of ketenebisthioacetals and trithio ortho esters from HF treatment of carboxylic acids is shown in Scheme 3. The initially formed thioester **6** undergoes acid-catalyzed addition of *p*-thiocresol, followed by elimination of $H_2O$ to the double adduct **8** or substitution by another *p*-thiocresol molecule to the trithio ortho ester **7**.

**Comparison of Hydrolysis Rates of *p*-Cresol and *p*-Thiocresol Esters 9a and 9b.** In contrast to the aryl ketone adducts formed through Friedel−Crafts acylation of anisole, *p*-cresol and *p*-thiocresol esters may permit hydrolysis to the carboxylic acid and in principle total recovery of the target peptide. As the *p*-cresol esters **2** preferentially cyclize to lactam under hydrolysis conditions, we chose the related model dipeptide, arginine-6-aminocaproic acid, to define appropriate conditions for the recovery of the target peptide. Arginine-6-aminocaproic acid was assembled on Merrifield resin and subjected to anhydrous HF treatment (1 h, 0 °C) in the presence of either *p*-cresol or *p*-thiocresol. The main components isolated from the resulting crude residues were characterized as the *p*-cresol ester **9a** or *p*-thiocresol ester **9b**, respectively (Scheme 4). As expected, an LC/MS profile of the *p*-thiocresol scavenged cleavage product indicated the presence of double and triple *p*-thiocresol adducts (data not shown). Only trace amounts of the free acid were isolated from either of the crude residues.

Hydrolysis of the purified esters **9a** and **9b** (Scheme 4) was slow at pH values below 10. However, the highly nucleophilic peroxide anion efficiently catalyzes the hydrolysis of the esters.[32] For instance, at pH 9 and using 1.2 equiv of hydrogen peroxide, ester hydrolysis was complete in 8 min. Interestingly, the catalyzed hydrolysis was found to proceed at comparable rates for both the oxyester **9a** and the thioester **9b** (data not shown). In terms of recovery of the carboxylic acid **10** from the respective esters, there was no advantage in choosing *p*-thiocresol over *p*-cresol as a scavenger.

**HF Cleavage of LTEN−Resin.** We have previously reported that during the HF/*p*-cresol cleavage of the tetrapeptide LTEN ($M_r$ 475) from phenylacetamidomethyl polystyrene resin (Pam),[33] a similar acylium ion-directed side reaction occurred.[34] Application of the low−high cleavage procedure[22] did not significantly alter the product distribution, *i.e.*, the *p*-cresol adducts were still formed in similar proportions. The yield of the *p*-cresol adduct ($M_r$ 565) increased with longer cleavage times (2 h) to 50%, after RP-HPLC isolation, but was strongly

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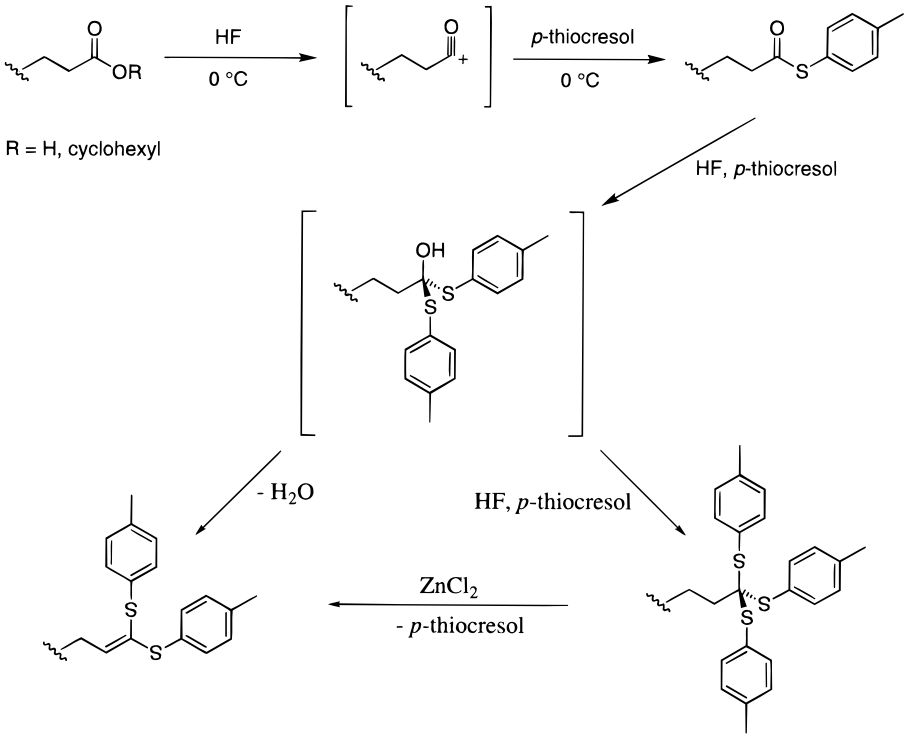
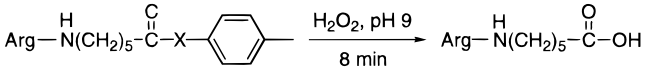
**Scheme 3.** Proposed Mechanism for the Formation of Ketenebisthioacetals and Trithio Ortho Esters under HF/*p*-Thiocresol Cleavage Conditions




**Scheme 4.** $H_2O_2$-Catalyzed Hydrolysis of *p*-Cresol Esters




**9a** X = O
**9b** X = S

**10**

influenced by the batch of resin used.[35a] LC/MS, $^1$H NMR (1D, COSY), and $^{13}$C NMR analyses allowed full characterization of the ester structure **11a** (Scheme 5). MS/MS of the ester molecular ion displayed a fragmentation pattern consistent with the proposed position of the ester, *i.e.*, on the glutamyl side chain. Contrary to the long-chain amino acids (Table 1), no ketone was observed in the crude product by NMR or LC/MS, even after longer cleavage times (2 h). Importantly, the unprotected tetrapeptide LTEN **16a** under identical HF cleavage conditions (HF/*p*-cresol (9:1) at 0 °C) generated the same products. Thus, similar to the long-chain acids, side chain deprotection of the glutamate residue in LTEN prior to high-HF treatment did not prevent acylium ion adduct formation.

**Side Reactions During Rearrangement of *N* → *O* Acyl Migrations.** In standard workup procedures, crude cleaved peptides are often treated with bicarbonate buffers (usually in the pH range of 7.5−9.5) for 12 h to reverse potential *N* → *O* shifts at threonine or serine residues.[23,39,40] In our hands, mild base treatments of the *p*-cresol ester **11a** gave complex mixtures. For example, LC/MS analysis[34,37] of ester **11a** at pH 8 (0.1 M $NH_4HCO_3$) and at pH 9.2 ($Na_2CO_3$/$NaHCO_3$ buffer), shown in Figure 1, indicated that in addition to the expected target peptide LTEN (and LTQN from aminolysis with ammonia at pH 8), three other main hydrolysis products were formed ($M_r$ 243, 232, and 214). Further LC/MS analysis at different time intervals (pH 9) revealed the existence of an intermediate with $M_r$ of 457. After 2 days at pH 9, the intermediate had fully converted to the hydrolysis products mentioned above. LTEN itself is stable under these basic conditions.

Scheme 5 depicts three possible reaction pathways for ester **11a**. On the basis of the observed intermediate and its conversion products, we believe reaction pathway **II** is the major contributor to hydrolysis of ester **11a**. Mild basic conditions promote the early formation of a pyrrolidinone **12** ($M_r$ 457). These imides react readily following several possible pathways depending on the buffer used and the pH of the solution. Intramolecular aminolysis via route **A** yields diketopiperazine **13** ($M_r$ 214) and pyrrolidinone **14** ($M_r$ 243). Hydrolysis via route **B** generates dipeptide **15** ($M_r$ 232) and pyrrolidinone **14**. Direct hydrolysis via route **C** on the other hand results in formation of the target LTEN **16a** (and in 0.1 M $NH_4HCO_3$, also LTQN **16b**). The absence of the dipeptide **15** at pH 8 is consistent with the proposed reaction scheme; at lower pH (data not shown) intramolecular aminolysis to diketopiperazine **13** will be preferred over bimolecular hydrolysis (by a hydroxide ion or ammonia). After 1 h of hydrolysis of ester **11a** at pH 9, the intermediate imide **12** was isolated by RP-HPLC and MS/MS analysis performed. The fragmentation pattern in the collision-induced dissociation experiment was consistent with the proposed structure. LC/MS analysis of a 2-day-old pH 9 solution of this purified imide **12** displayed a similar hydrolysis profile: hydrolysis to dipeptide **15**, diketopiperazine **13**, and some recovery of LTEN **16a**. The existence of these side

(35) (a) In another experiment using a different batch of Boc-Asn-$OCH_2$-Pam resin from the same company, the yield of the *p*-cresol adduct under the same reaction conditions was significantly lower (10%). (b) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882−899.

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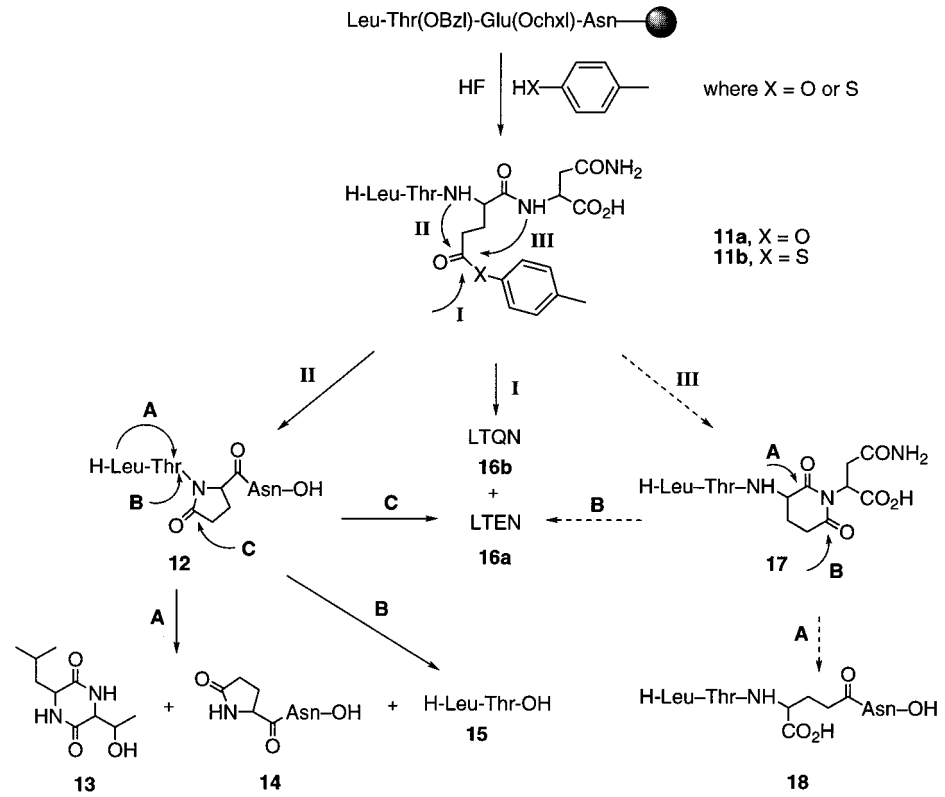
**Scheme 5.** Reaction Pathways of Mild Base Hydrolysis of LTEN *p*-Cresol Ester **11a**




products was confirmed by ES-MS and RP-HPLC coelution experiments with authentic samples.

It is conceivable that the ester **11a** partially cyclizes to a six-membered ring system **17**, with molecular weight of 457, through nucleophilic attack by the nitrogen atom of the $(i + 1)$ residue. Further hydrolysis of **17** could produce LTEN (**16a**) or the side chain connected $\delta$-peptide LTE(N) **18** ($M_r$ 475). The latter product however was not detected. From the observed product distributions in several hydrolysates of ester **11a**, it is clear that reaction route **II** via the imide **12** is the major contributor in the hydrolysis, thus limiting the significance of route **III**, if present at all.

**Minimizing Side Reactions During Rearrangement of *N* → *O* Acyl Migrations.** Clearly, the use of $NH_4HCO_3$ buffer (pH 8) for 12 h to reverse potential $N \rightarrow O$ shifts[23,39,40] induced a string of side reactions at *p*-cresol-esterified glutamyl residues, generating both side chain amide analogues (*i.e.*, Glu→Gln) of the target peptide and several fragments. The purity and yield of the final product was thus significantly reduced. To avoid these fragmentation reactions, and recover LTEN, we applied a $H_2O_2$-catalyzed hydrolysis of isolated ester **11a** (Figure 2). At pH 10, treatment with 2 equiv of $H_2O_2$ for 5 min produced the target tetrapeptide **16a** with minor amounts of dipeptide **15** formed. After treatment with 25 equiv $H_2O_2$ at pH 10 for 5 min, no contaminants were detected and homogeneous **16a** was readily recovered in excellent yield.[41a]

When *p*-cresol was replaced by *p*-thiocresol in the HF cleavage procedure, the corresponding *p*-thiocresol ester ($M_r$ 581) was isolated in yields similar to those of the *p*-cresol ester. In a mixture of 1:1 *p*-cresol/*p*-thiocresol, the thioester **11b** was formed preferentially over the oxyester. Further LC/MS analysis of both cleavage mixtures (data not shown) indicated again the presence of "double" ($M_r$ 687) and "triple" ($M_r$ 811) *p*-thiocresol adducts. In contrast to the facile peroxide ion-catalyzed hydrolysis of the cresol esters, the double and triple adducts were stable under these conditions and did not lead to recovery of the target peptide (data not shown).

**Application to Multi-Glu-Containing Peptides: Synthesis of MRP14(89-114).** HF-induced *p*-cresol ester formation and subsequent side reactions induced by mild base are not confined to the LTEN sequence. To further demonstrate the significance of these findings with respect to obtaining homogeneous product after HF treatment, we carried out a detailed study on the synthesis and HF cleavage of MRP14(89-114) (**19**), also known as neutrophil immobilization factor (NIF).[41b,42] The sequence contains four glutamyl residues that are sites for HF-induced acylium ion formation (Table 2).

ASH**E**KMH**E**GD**E**GPGHHHKPGLG**E**GTP $M_r$ 2728.9
**19**

The assembly of the peptide was performed on Pam resin using in situ Boc/HBTU activation protocols.[43] The peptide was detached from the resin with anhydrous HF (0 °C, 1 h) in the presence of 10% *p*-cresol, *p*-cresol/*p*-thiocresol (1:1), or *p*-thiocresol. The LC/MS profiles of the three crude products are shown in Figure 3.

(41) (a) It is not inconceivable that the proposed peroxide-catalyzed hydrolysis of *O*-esters could lead to chain cleavage at Ser/Thr residues in $N \rightarrow O$ acyl rearranged peptides. To examine this in more detail, we synthesized and isolated $N \rightarrow O$ acyl rearranged human immunodeficiency virus-1 proteinase, HIV-1 PR(81-99) (P. F. Alewood, unpublished results). The product was then subjected to the described $H_2O_2$-catalyzed hydrolysis conditions. No chain cleavage at Ser/Thr residues in HIV-1 PR(81-99) was observed by HPLC/MS analysis and pure HIV-1 PR(81-99) was recovered in quantitative yields. Thus reversal of the $N \rightarrow O$ acyl migration under our proposed conditions occurs preferentially to peroxide-catalyzed cleavage. (b) Odink, K.; Cerletti, N.; Brueggen, J.; Clerc, R. G.; Tarcsay, L.; Zwadlo, G.; Gerhards, G.; Schlegel, R.; Sorg, C. *Nature* **1987**, *330*, 80−82.

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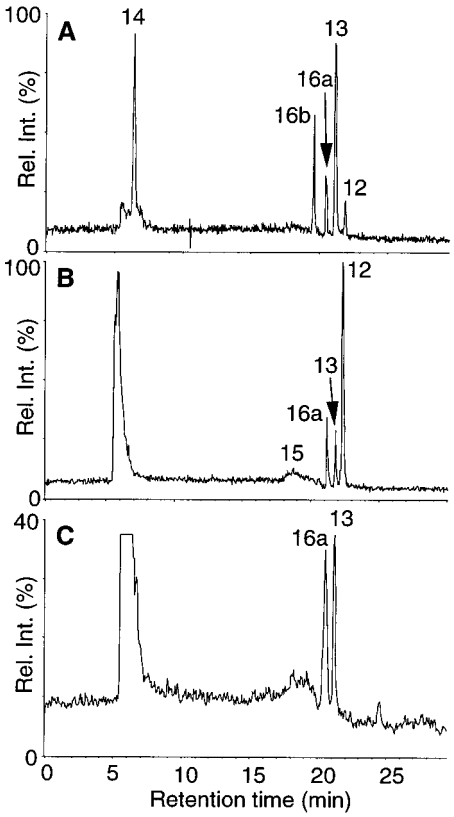


**Figure 1.** LC/MS analyses of a buffered solution of *p*-cresol ester **11a** (A) after 2 days in 0.1 M $NH_4HCO_3$ buffer (pH 8), (B) after 1 h in 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.2), and (C) after 2 days in 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.2). The products were separated on a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) column using a linear 0−60% buffer B gradient over 60 min at a flow rate of 150 $\mu$L/min and directly infused into the mass spectrometer using Turbo Ionspray. Full scan mass spectra were acquired over the mass range of 200−1000 with a scan step of 0.1 Da.

With *p*-cresol as the sole scavenger (Figure 3A), four major side products of the target peptide were detected. Four peaks displayed an *m*/*z* value corresponding to the formation of a single cresol ester ($M_r$ 2819 (2729 + 90)) at each glutamyl residue, **20a**−**d** (Table 2). All *p*-cresol esters were separated from the target peptide and combined represented 21% of the total products. Importantly, HF/*p*-cresol treatment of purified MRP14-(89-114) produced a comparable LC/MS profile. Application of the low−high HF cleavage procedure did not prevent the formation of *p*-cresol and *p*-thiocresol adducts, since glutamyl acylium ions can be generated from either the protected or free glutamyl residues.

Cleavage using HF/*p*-thiocresol (9:1) significantly increased the number of side products in the crude cleavage product as observed by LC/MS analysis (Figure 3B). At least 12 side products could be readily characterized, four peaks with a $M_r$ of 2835 corresponding to the four single *p*-thiocresol adducts (2729 + 106 Da). Four peaks corresponding to a $M_r$ of 2941 (2729 + 212) indicated the formation of four double *p*-thiocresol adducts. On the basis of our earlier results with aminocaproic acid, we propose that these double *p*-thiocresol adducts originate from the formation of a ketenebisthioacetal at each glutamyl residue rather than the formation of thioesters at two distinct Glu sites. A further four peaks each displayed *m*/*z* values corresponding to 2941 and 3065. $M_r$ 3065 represents the molecular weight of the NIF sequence containing a single trithio ortho ester (2729 + 336). As is the case for the previously described trithio ortho esters, these triple thiocresol adducts are

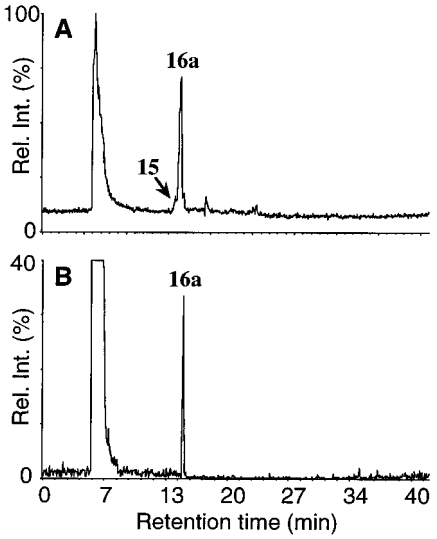


**Figure 2.** LC/MS analyses of catalyzed hydrolysis of isolated LTEN *p*-cresol ester **11a** in pH 10 $Na_2CO_3/NaHCO_3$ buffer for 8 min using (A) 2 equiv of $H_2O_2$ and (B) 25 equiv of $H_2O_2$. The products were separated on a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) column using a linear 0−60% buffer B gradient over 30 min at a flow rate of 150 $\mu$L/min and directly infused into the mass spectrometer using Turbo Ionspray. Full scan mass spectra were acquired over the mass range of 300−1000 Da with a scan step of 0.1 Da.

**Table 2.** Molecular Mass Comparison of the *p*-Cresol Esters of MRP14(89-114) (**19**), with Those Observed by LC/MS Analysis of the Crude Material after HF/*p*-Cresol (9:1) Treatment of Resin-Bound MRP14(89-114) Where **X** Indicates a *p*-Cresol Ester on the Glutamyl Side Chain

| Observed $M_r$ (Fig. 3A) | Possible sites of *p*-cresol ester formation on **19** | Cleavage product | Calculated $M_r$ |
|---|---|---|---|
| 2728.7 | ---**E**---**E**--**E**----------**E**--- | **19** | 2728.9 |
| 2819.5 | ---**X**---**E**--**E**----------**E**--- | **20a** | 2819.0 |
| 2819.1 | ---**E**---**X**--**E**----------**E**--- | **20b** | 2819.0 |
| 2819.5 | ---**E**---**E**--**X**----------**E**--- | **20c** | 2819.0 |
| 2819.1 | ---**E**---**E**--**E**----------**X**--- | **20d** | 2819.0 |

expected to release one thiocresol molecule (−124) under Turbo Ionspray LC/MS conditions.[44] Consequently, we believe that the 2941 and 3065 *m*/*z* ions in these LC/MS peaks originate mainly, if not exclusively, from the trithio ortho ester derivatives of **19**. After RP-HPLC purification of MRP14(89-114) all cleavage side products combined represented 29% of the total quantity of isolated material.

When equimolar amounts of *p*-cresol and *p*-thiocresol were used in the HF cleavage (Figure 3C), the yield of side products increased to 33%. The composition of the side products was complex with molecular weights corresponding to *p*-cresol and *p*-thiocresol esters (2729 + 90 and 2729 + 106), ketenebisthio-acetals (2729 + 212), a combination of ketenebisthioacetals and *p*-cresol ester (2729 + 302), and trithio ortho esters (2729 + 336).

**Mild Base Treatment of Crude MRP14(89-114).** Similar to LTEN, mild base treatment (0.1 M $NH_4HCO_3$, pH 8.5) of

(44) Fractions from the preparative RP-HPLC separation of crude thiocresol adducts were found to have a single mass of 3064 (triple thiocresol adduct) by ES-MS without Turbo Ionspray. However, when LC/MS conditions were simulated by employing Turbo Ionspray on those fractions, both 2942 and 3064 *m*/*z* ions were observed.

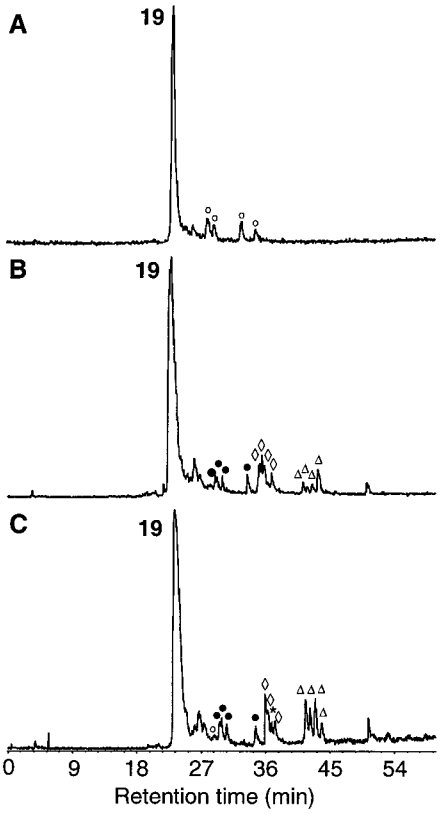


**Figure 3.** LC/MS analyses of the crude HF cleavage products of resin-bound MRP14(89-114) **19** using (A) HF/*p*-cresol (9:1), (B) HF/*p*-thiocresol (9:1), and (C) HF/*p*-cresol/*p*-thiocresol (18:1:1). The crude products were dissolved in aqueous 0.1% TFA (1 mg/mL) and then loaded directly onto a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) HPLC column. The products were separated using a linear 0−60% buffer B gradient over 60 min at a flow rate of 150 $\mu$L/min and directly infused into the mass spectrometer using Turbo Ionspray. Full scan mass spectra were acquired over the mass range of 400−2000 with a scan step of 0.1 Da. The symbols in the figure indicate peaks of the following molecular weights, where $M = 2728.9$ Da: (○) $M + 90.1$ Da; (●) $M + 106.0$ Da; (◇) $M + 212$ Da; (*) $M + 303$ Da; (△) $M + 212$ and $M + 336$ Da.

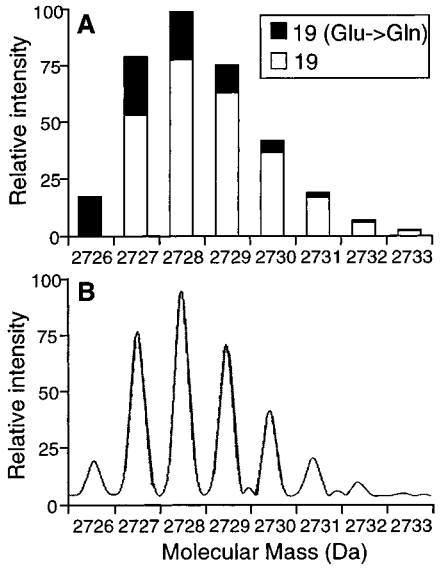


**Figure 4.** (A) Theoretical isotope distribution for the molecular mass region of a 3:1 mixture of MRP14(89−114) **19** and its single Glu → Gln mutant. (B) The molecular mass region of the reconstructed FT-ICR MS spectrum of purified (RP-HPLC) MRP14(89-114) obtained from mild base treatment (0.1 M $NH_4HCO_3$ (pH 8.5) of crude HF/*p*-cresol cleaved product.

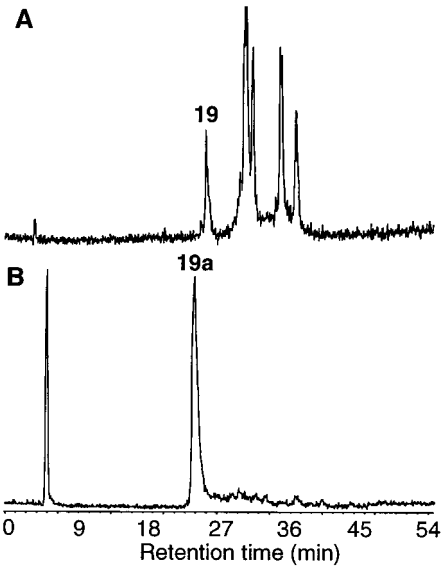


**Figure 5.** LC/MS analyses of (A) separated and combined *p*-cresol adducts **20a**−**d**; (B) *p*-cresol adducts treated for 30 min with 5% $H_2O_2$ in 0.1 M $NaHCO_3$ (pH 8.5). The solutions were acidified (pH 2) and were loaded directly onto a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) HPLC column. The products were separated using a linear 0−60% buffer B gradient over 60 min at a flow rate of 150 $\mu$L/min and directly infused into the mass spectrometer using Turbo Ionspray. Full scan mass spectra were acquired over the mass range of 400−2000 with a scan step of 0.1 Da.

crude MRP14(89-114) (HF/*p*-cresol; 9:1) resulted in partial amidation of the *p*-cresol esterified Glu residues (*i.e.*, Glu to Gln) and fragmentation. RP-HPLC separation of our target peptide from these Glu to Gln mutants was not readily achieved. ES-MS analysis of the purified product did not provide sufficient resolution to unambiguously confirm presence of Glu to Gln mutants (1 Da difference). However, the presence of Gln mutants at each Glu site was unequivocally confirmed by Edman sequencing of the MRP14(89-114) separated from the $NH_4HCO_3$ solution. Further, the high-resolution FT-ICR MS deconvoluted spectrum of the same fraction (Figure 4) displayed an isotope envelope corresponding to a (1:3) mixture of Glu to Gln mutants over target peptide **19**. Even though these impurities were present in significant quantity, it is conceivable they would have remained unnoticed after standard workup, purification, and characterization by RP-HPLC and low-resolution ES-MS analysis.

To examine the fragmentation pathways in detail, the *p*-cresol adducts were isolated, combined, treated with 0.1 M $NH_4HCO_3$ pH 8.5 for 24 h, and then analyzed by LC/MS (Figure 6). RP-HPLC analysis demonstrated that the target peptide coeluted with several smaller fragments. Scheme 6 depicts the predicted hydrolysis pathways for *p*-cresol-esterified peptides in a mild base buffer, including formation of the free acid, amidation (Glu to Gln), and fragmentation through an intermediate pyrrolidinone. In Table 3 we have listed the corresponding fragments for peptides **20a**−**d** (fragmentation pathway **IIB**) and compared the theoretical molecular weights with the observed masses in the LC-MS analysis (Figure 6). The experimental data are in good agreement with the proposed fragmentation route **IIB**. Furthermore, the fact that all four *p*-cresol esters (**20a**−**d**) are readily hydrolyzed eliminates the possibility that they derive from incomplete Bzl-deprotection at threonine or serine. Treatment of the *p*-cresol adducts with a $NaHCO_3/Na_2CO_3$ buffer

**Scheme 6.** General Routes for the Degradation of Peptides Containing *p*-Cresol-Esterified Glutamyl Residues at pH 8

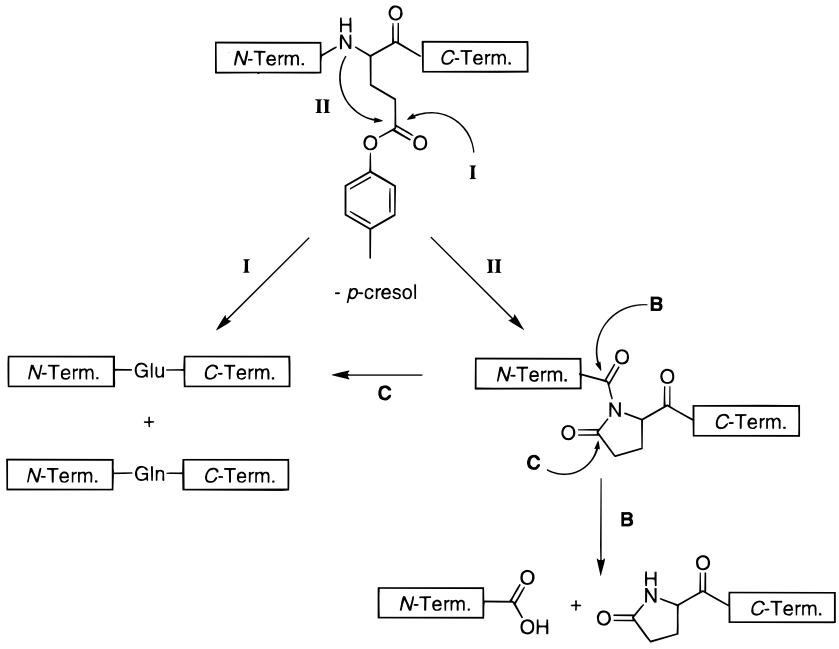


**Table 3.** Comparison of Predicted Fragments of Peptides **20a**−**d** Formed after Base Treatment via Pathway **IIB** with Molecular Masses Observed in the LC/MS Analysis of the Crude Product Shown in Figure 6

| fragments of **20a**−**d**[a] | | calcd $M_r$ | | found $M_r$ | |
|---|---|---|---|---|---|
| *N*-term.[b] | *C*−term.[b] | *N*-term. | *C*-term. | *N*-term. | *C*-term. |
| ASH | BKMHEGDEGPGHHHKPGLGEGTP | 313.1 | 2415.6 | 313.2 (**A**) | 2414.9 (**H**) |
| ASHEKMH | BGDEGPGHHHKPGLGEGTP | 838.9 | 1890.0 | 838.5 (**B**) | 1889.5 (**G**) |
| ASHEKMHEGD | BGPGHHHKPGLGEGTP | 1140.2 | 1588.7 | 1140.0 (**D**) | 1588.2 (**F**) |
| ASHEKMHEGDEGPGHHHKPGLG | BTGP | 2344.5 | 384.4 | 2344.1 (**E**) | 384.0 (**C**) |

[a] B denotes pyroglutamic acid. [b] *N*- and *C*-term. refer to *N*- and *C*-terminal fragments, respectively.

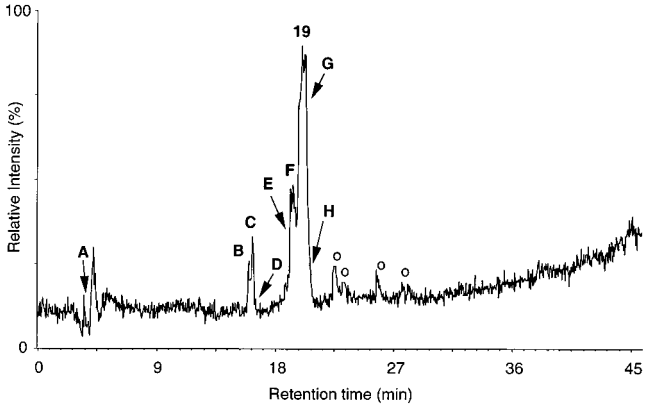


**Figure 6.** Detailed LC/MS analysis of *p*-cresol adducts **20a**−**d** treated with 0.1 M $NH_4HCO_3$ (pH 8.5) for 24 h. The solution was loaded directly onto a Vydac reversed phase C-18 (5 $\mu$m, 0.21 × 25 cm) HPLC column. The products were separated using a linear 0−60% buffer B gradient over 60 min at a flow rate of 150 $\mu$L/min and directly infused into the mass spectrometer using Turbo Ionspray. Full scan mass spectra were acquired over the mass range of 400−2000 with a scan step of 0.1 Da. The letters in the figure indicate fragments of **20a**−**d** from Table 1, while the symbol (○) denotes peaks with a molecular weight corresponding to the initial single *p*-cresol adduct.

(pH 9.2) avoided formation of Gln mutants but resulted in a similar fragmentation pattern.

In a similar fashion the combined *p*-thiocresol adducts were subjected to mild base treatment in a 0.1 M $NaHCO_3$ buffer at pH 8.5 for 24 h and analyzed by LC/MS (data not shown). As with hydrolysis of the *p*-cresol adducts base treatment of the *p*-thiocresol adduct mixture led to fragmentation according to route **IIB**. The double and triple adducts observed in the original mixture were stable to these conditions.

**Efficient Recovery of Target Peptide MRP14(89-114).** To study the feasibility of recovery of target peptide, we initially investigated the efficacy of the $H_2O_2$ catalysis conditions on the isolated *p*-cresol and *p*-thiocresol adducts. The separated *p*-cresol esters converted nearly quantitatively to the methionine-oxidized MRP14(89-114) (**19a**), as illustrated in the LC/MS profile (Figure 5B). By comparison the $H_2O_2$-catalyzed hydrolysis of the separated *p*-thiocresol adducts (esters, ketenebisthioacetals, and trithio ortho esters) was less effective with the ketenebisthioacetals and trithio ortho esters proving resistant to $H_2O_2$-catalyzed hydrolysis. Further studies on the crude HF/*p*-cresol cleavage product of **19** were undertaken. Clean hydrolysis of the *O*-esters was observed as shown in Figure 7. Subsequent reduction of the oxidized methionine was quantitative with *N*-mercaptoacetamide under a nitrogen atmosphere to give highly homogeneous MRP14(89-114).

## Conclusion

The advent of chemical ligation techniques has opened new routes to the chemical synthesis of proteins.[45−52] These advances have largely relied upon successful Boc SPPS to

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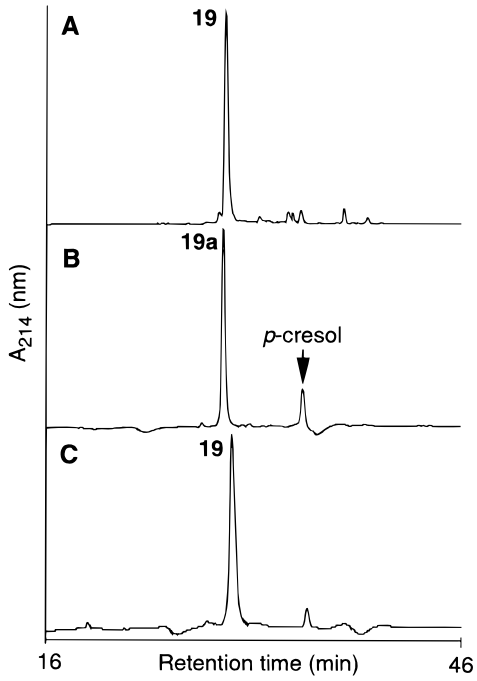


**Figure 7.** HPLC analysis of crude MRP14(89-114) HF/*p*-cresol cleavage product (A) untreated, (B) treated with 5% $H_2O_2$ in 0.1 M $NaHCO_3$ (pH 8.5) for 10 min, and (C) followed by reduction with 15% acetic acid and 10% *N*-mercaptoacetamide under $N_2$ at 37 °C for 18 h. The solutions were acidified (pH 2) and were loaded directly onto a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) HPLC column. The products were separated using a linear 0−60% buffer B gradient over 60 min at a flow rate of 1 mL/min.

deliver highly homogeneous peptides typically 50+ residues. In this work we have addressed the long-standing "acylium ion" problem which has plagued many synthetic efforts in the past. The new chemical insights describe not only the nature of the problem more fully but have also facilitated the development of novel chemical procedures that largely overcome the former difficulties. To synthesize homogeneous peptides containing long-chain acids or glutamyl residues, we recommend that *p*-cresol be utilized as a reversible acylium ion scavenger during HF cleavage. Where feasible, *p*-thiocresol should not be used either alone or in combination with *p*-cresol. Furthermore, mild base treatments on crude cleaved peptide (typically employed to reverse potential $N \rightarrow O$ shifts) should be replaced by a mild peroxide-catalyzed hydrolysis step. The implementation of this novel concept should help facilitate further advances in the chemical synthesis of highly homogeneous proteins.

## Experimental Section

**Materials and Methods.** Merrifield resin (*s.v.*:4.1 mmol/g), $N_\alpha$-*tert*-butoxycarbonyl (Boc)-L-amino acids, and reagents used during chain assembly were peptide synthesis grade purchased from Auspep (Melbourne, Australia), Novabiochem (San Diego, CA), and the Peptide Institute (Osaka, Japan). Boc-L-amino acid-$OCH_2$ (Pam) resin was purchased from Applied Biosystems (Foster City, CA). Dichloromethane (DCM), diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Auspep (Melbourne, Australia). *p*-Cresol, *p*-thiocresol, hydrogen peroxide, 6-aminocaproic acid, and 4-aminobutyric acid were purchased from Aldrich or Fluka (Sydney, Australia). HPLC grade acetonitrile was purchased from Millipore-Waters (Sydney, Australia). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Richelieu Biotechnologies (Quebec, Canada). Deionized water was used throughout and was prepared with a Milli-Q water purification system (Millipore-Waters). Screw-cap glass peptide synthesis reaction vessels (20 mL) with sintered glass filter frits were obtained from Embell Scientific Glassware (Queensland, Australia). An all-Kel-F apparatus (Peptide Institute) was used for HF cleavage. Argon, helium, and nitrogen (all ultrapure grade) were from BOC Gases (Queensland, Australia). $^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian 300 MHz Gemini in $CD_3OD$, and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$. Reversed phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters 600E solvent delivery system equipped with a 484 nm absorbance detector and recorded on an Apple Macintosh computer using Model 600 software (Applied Biosystems Inc.). RP-HPLC was performed using detection wavelengths of 214 and 230 nm on Vydac C-18 analytical (5 $\mu$m, 0.46 cm × 25 cm) and preparative C-18 (10 $\mu$m, 2.2 cm × 25 cm) columns, respectively. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% $CH_3CN$, 10% $H_2O$, 0.09% TFA) over 60 min at a flow rate of 1 mL/min (analytical) and 8 mL/min (preparative).

**Mass Spectrometry.** Mass spectra were acquired on a PE-Sciex API-III triple-quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 $\mu$L) were injected into a moving solvent (30 $\mu$L/min; 50:50 $CH_3CN$/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 $\mu$m i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100−120 $\mu$m diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range of 400−2000 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed *m*/*z* values using the MacSpec 3.3 and Biomultiview 1.2 software packages (PE-Sciex Toronto, Canada). Calculated theoretical monoisotopic and average masses were determined using the MacBiospec program (PE-Sciex Toronto, Canada). LC/MS runs were carried out using a linear gradient on a 140B ABI dual syringe pump solvent delivery system and a Vydac reversed phase C-18 (5 $\mu$m, 300 Å, 0.21 × 25 cm) column at a flow rate of 150 $\mu$L/min. Samples (typically 5 $\mu$L of 1 mg/mL solution) were loaded directly on the column, and the eluent was directly connected to the mass spectrometer via a 30 cm, 75 $\mu$m i.d. fused silica capillary. The application of Turbo Ionspray (5 L/min $N_2$ at 500 °C) allowed the introduction of the total eluent without splitting and loss in sensitivity. Acquisition parameters were as described above. For tandem mass spectrometry the molecular ions were bombarded in quadrupole-2 with argon; the collision cell gas thickness was $3.5 \times 10^{14}$ atoms/cm$^2$, and the collision energy was 10−20 eV. Quadrupole-3 was then scanned to monitor product ions from *m*/*z* 50 to just above the parent ion molecular mass in 3 s with a step size of 0.2 Da. Accurate mass measurement of compound **5a** was performed on a Kratos MS25 mass spectrometer. Other high-resolution data were obtained on a Bruker Spectrospin BioAPEX external-ion-source Fourier transform electrospray mass spectrometer at a magnetic field of 4.7 T. Typically samples were dissolved in 50% aqueous methanol containing 1% acetic acid.

**Peptide Synthesis.** 6-Aminocaproic acid and 4-aminobutyric acid were Boc protected and coupled to Merrifield resin using standard procedures.[53,54] LTEN and MRP14(89-114) were chemically synthesized stepwise using *in situ* neutralization/HBTU activation protocols for Boc chemistry as previously described.[43] The synthesis was performed on Boc-Asn(xanthyl)-$OCH_2$ Pam (or Merrifield resin) and Boc-Pro-$OCH_2$ Pam resin, respectively. The following amino acid side chain protection was used: Boc-Asn(xanthyl)-OH, Boc-Asp(*O*-cyclohexyl)-OH, Boc-Glu(*O*-cyclohexyl)-OH, Boc-His($N_{im}$-2,4-dinitrophenyl)-OH, Boc-Lys-($N_\epsilon$-2-chlorobenzyloxycarbonyl)-OH, Boc-Ser(*O*-

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benzyl)-OH, and Boc-Thr(*O*-benzyl)-OH. Each residue was coupled for 10 min, and coupling efficiencies determined by the quantitative ninhydrin reaction.[55] The average yield of chain assembly was 99.75% for LTEN and 99.85% for MRP14(89-114). Before HF cleavage, the dinitrophenyl groups were removed by thiolysis (20% $\beta$-mercaptoethanol, 10% DIEA, and 70% DMF; 2 × 2 h) prior to *N*-terminal Boc deprotection with 100% TFA. The resin was then washed with DCM and dried under nitrogen.

**HF Cleavage.** Peptide resin (300 mg) was treated at the given temperature with 10 mL of HF/*p*-cresol (9:1 (v/v)), HF/*p*-thiocresol (9:1 (v/v)), or HF/*p*-cresol/*p*-thiocresol (18:1:1 (v/v)). After evaporation of the HF, the crude product was precipitated and washed with cold diethyl ether (2 × 10 mL), dissolved in 20% aqueous acetic acid (5 mL), and lyophilized after aqueous dilution.

**4-Aminobutanoic acid 4′-methylphenyl ester, TFA salt (2a):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.19 (d, $J$ = 8.5 Hz, 2H, *H*-ar), 6.95 (d, $J$ = 8.5 Hz, 2H, *H*-ar), 3.05 (t, $J$ = 7.8 Hz, 2H, C$H_2$−N), 2.73 (t, $J$ = 7.1 Hz, 2H, C$H_2$ −C═O), 2.33 (s, 3H, C$H_3$), 2.04 (m(5), 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 173.03 (*C*═O), 150.04 (C-*i*-ar), 136.93 (C-*p*-ar), 131.00 (C-*m*-ar), 122.44 (C-*o*-ar), 40.09 (*C*$H_2$−N), 31.77 (*C*$H_2$−CO), 23.84 (*C*$H_2$), 20.99 (*C*$H_3$); ES-MS $M_r$ 193.1, calcd for $C_{11}H_{15}NO_2$, 193.1 (monoisotopic).

**6-Aminohexanoic acid 4′-methylphenyl ester, TFA salt (2b):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.18 (d, $J$ = 8.7 Hz, 2H, *H*-ar), 6.93 (d, $J$ = 8.5 Hz, 2H, *H*-ar), 2.94 (t, $J$ = 7.8 Hz, 2H, C$H_2$−N), 2.60 (t, $J$ = 7.1 Hz, 2H, C$H_2$ −C═O), 2.32 (s, 3H, C$H_3$), 1.74 (m(7), 4H, 2 × C$H_2$), 1.51 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 173.87 (*C*═O), 150.00 (C-*i*-ar), 136.69 (C-*p*-ar), 130.85 (C-*m*-ar), 122.34 (C-*o*-ar), 40.56 (*C*$H_2$−N), 34.61 (*C*$H_2$−CO), 28.29 (*C*$H_2$), 26.86 (*C*$H_2$), 25.34 (*C*$H_2$), 20.84 (*C*$H_3$), ES-MS $M_r$ 221.1, calcd for $C_{13}H_{19}NO_2$, 221.1 (monoisotopic).

**6-Amino-1-(5′-methyl-2′-hydroxyphenyl)hexan-1-one, TFA salt (4b):** $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 207.98 (*C*═O), 161.22 (*C*-ar), 138.31 (*C*-ar), 131.09 (*C*-ar), 129.56 (*C*-ar), 120.40 (*C*-ar), 118.85 (*C*-ar), 38.80 (*C*$H_2$−N), 34.60 (*C*$H_2$−C═N), 28.43 (*C*$H_2$), 27.03 (*C*$H_2$), 24.65 (*C*$H_2$); ES-MS $M_r$ 221.1, calcd for $C_{13}H_{19}NO_2$, 221.1 (monoisotopic).

**2-(4,5′-Dihydro-3*H*-pyrrol-2′-yl)-4′-methylphenol, TFA salt (5a):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.613 (d, $J$ = 2.0 Hz, 1H, *H*-ar), 7.50 (dd, $J^1$ = 2.0 Hz, $J^2$ = 8.6 Hz, 1H, *H*-ar), 7.03 (d, $J$ = 8.6 Hz, 1H, *H*-ar), 4.18 (t, $J$ = 7.7 Hz, 2H, C$H_2$−N), 3.61 (t, $J$ = 8.3 Hz, 2H, C$H_2$−C═N), 2.34 (s, 3H, C$H_3$−Phe), 2.33 (m(5), 2H, CH$_2$−C$H_2$−CH$_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 183.22 (*C*═N), 159.84 (*C*-ar), 140.77 (*C*-ar), 134.40 (*C*-ar), 131.62 (*C*-ar), 117.93 (*C*-ar), 113.67 (*C*-ar), 55.02 (*C*$H_2$−N), 36.38 (*C*$H_2$−C═N), 20.31 (*C*$H_3$), 20.23 (CH$_2$−*C*H$_2$−CH$_2$); ES-MS $M_r$ 175.1, accurate mass EI obsd 175.0997, calcd for $C_{11}H_{13}NO$, 175.0997 (monoisotopic).

**(1′-[2′-Hydroxy-5′-methylphenyl])-2-azacyclohept-1-ene, TFA salt (5b):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.41 (s, 1H, *H*-ar), 7.16 (dd, 1H, $J_o$ = 8.6 Hz, $J_m$ = 2.3 Hz, *H*-ar), 6.68 (d, $J$ = 8.7 Hz, 1H, *H*-ar), 3.81 (m, 2H, C$H_2$), 3.12 (m, 2H, C$H_2$), 2.23 (s, 3H, C$H_3$), 1.94 (m, 2H, C$H_2$), 1.72 (m, 4H, C$H_2$); $^{13}$C NMR (75 MHz, $CDCl_3$, ppm) $\delta$ 183.27 (*C*═N), 173.24 (*C*-ar), 138.48 (*C*-ar), 130.90 (*C*-ar), 129.70 (*C*-ar), 124.72 (*C*-ar), 123.49 (*C*-ar), 46.97 (*C*$H_2$−N═C), 31.50 (*C*$H_2$), 28.42 (*C*$H_2$), 28.10 (*C*$H_2$), 24.43 (*C*$H_2$), 20.72 (*C*$H_3$); ES-MS $M_r$ 203.1, calcd for $C_{13}H_{17}NO$, 203.1 (monoisotopic).

**6-Aminohexanethioic acid *S*-4′-methylphenyl ester, TFA salt (6):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.26 (s, 4H, *H*-ar), 2.92 (t, $J$ = 7.5 Hz, 2H, C$H_2$−N), 2.71 (t, $J$ = 7.2 Hz, 2H, C$H_2$−C═N), 2.37 (s, 3H, C$H_3$), 1.71 (m, 4H, 2 × C$H_2$), 1.45 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 199.54 (*C*═S), 141.20 (*C*-ar), 135.75 (*C*-ar), 131.12 (*C*-ar), 125.81 (*C*-ar), 43.92 (*C*$H_2$−N), 40.67 (*C*$H_2$−C═S), 28 (*C*$H_2$), 27.6 (*C*$H_2$), 26.20 (*C*$H_2$), 21.42 (*C*$H_3$); ES-MS $M_r$ 236.9, calcd for $C_{13}H_{19}NOS$, 237.1 (monoisotopic).

**1,1,1-Tris-(4′-methylphenyl)sulfanyl-6-aminohexane, TFA salt (7):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.51 (d, $J$ = 7.6 Hz, 6H, *H*-ar), 7.17 (d, $J$ = 7.6 Hz, 6H, *H*-ar), 2.81 (m, 2H, C$H_2$−N), 2.35 (s, 9H, C$H_3$), 1.75 (m, 2H, C$H_2$), 1.65 (m, 2H, CH$_2$), 1.52 (m, 2H, C$H_2$), 1.10 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 142.43, 139.07, 131.92, 131.37, 79.14, 42.52, 42.20, 30.04, 28.79, 27.41, 22.88; ES-MS $M_r$ 467.0, calcd for $C_{27}H_{33}NS_3$, 467.2 (monoisotopic).

**1,1-Bis(4-methylphenyl)sulfanyl-6-aminohex-1-ene (8):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.12 (s, 4H, *H*-ar), 7.09 (s, 4H, *H*-ar), 6.17 (t, $J$ = 6.5 Hz, 1H, *H*-C═C), 2.91 (m, 2H, C$H_2$−N), 2.46 (m, 2H, C$H_2$−C═N), 2.31 (s, 3H, C$H_3$), 2,30 (s, 3H, C$H_3$), 1.66 (m, 2H, CH$_2$), 1.50 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$, ppm) $\delta$ 142.78 (*C*H), 137.43 (*C*), 136.58 (*C*), 131.36 (*C*H), 129.95 (*C*H), 129.82 (*C*H), 129.78 (*C*H), 129.00 (*C*), 38.56 (*C*$H_2$), 30.24 (*C*$H_2$), 26.66 (*C*$H_2$), 25.31 (*C*$H_2$), 20.72 (*C*$H_3$), 20.64 (*C*$H_3$); ES-MS $M_r$ 343.1, calcd for $C_{20}H_{25}NS_2$, 343.1 (monoisotopic).

**Arginine-6-aminocaproic acid 4′-methylphenyl ester, TFA salt (9a):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 6.95 (d, 2H, *H*-ar), 6.76 (d, 2H, *H*-ar), 3.77 (t, 1H, C$\alpha$), 3.16 (m, 3H, C$H_2$), 3.04 (m, 3H, C$H_2$), 2.42 (t, 2H, C$H_2$), 2.13 (m, 2H, C$H_3$), 1.72 (m, 4H, C$H_2$), 0.47 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 173.52 (*C*═O), 170.00 (*C*═O), 158.71 (*C*-ar), 149.96 (*C*-ar), 136.70 (*C*-ar), 130.83 (*C*-ar), 122.36 (*C*-ar), 54.11 (*C*H), 41.68 (*C*$H_2$), 39.97 (*C*$H_2$), 39.74 (*C*$H_2$), 32.17 (*C*$H_2$), 29.17 (*C*$H_2$), 25.63 (*C*$H_2$), 25.56 (*C*$H_2$), 25.39 (*C*$H_2$), 20.84 (*C*$H_3$); ES-MS $M_r$ 377.2, calcd for $C_{19}H_{31}N_5O_3$, 377.2 (monoisotopic).

**Arginine-6-aminocaprothioic acid *S*-4′-methylphenyl ester, TFA salt (9b):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.07 (s, 4H, *H*-ar), 3.75 (t, 1H, C$\alpha$), 3.11 (m, 4H, C$H_2$), 3.02 (m, 2H, C$H_2$), 2.53 (t, 2H, C$H_2$), 2.16 (s, 3H, C$H_3$), 1.68 (m, 4H, CH2), 1.48 (m, 2H, C$H_2$); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 199.07(*C*═S), 169.92 (*C*═O), 158.71 (*C*$\epsilon$), 141.07 (*C*-ar), 135.61 (*C*-ar), 130.98 (*C*-ar), 125.50 (*C*-ar), 54.08 (*C*$\alpha$), 41.69 (*C*H$_2$), 41.44 (*C*H$_2$), 39.75 (*C*H$_2$), 29.69 (*C*H$_2$), 26.09 (*C*H$_2$), 25.39 (*C*H$_2$), 21.27 (*C*H$_3$); ES-MS $M_r$ 393.1, calcd for $C_{19}H_{31}N_5O_2S$, 393.2 (monoisotopic).

**Comparison of Hydrolysis Rates of 9a and 9b.** Fifty microliters of a 10 mg/mL solution of the ester in water was diluted with 450 $\mu$L of a pH 9.2 buffer ($Na_2CO_3$/$NaHCO_3$), and 12.5 $\mu$L of an 0.3% hydrogen peroxide solution in water was immediately added. Aliquots of 50 $\mu$L were taken out after specific time intervals (1, 2, 4, and 8 min), and the hydrolysis was quenched with 100 $\mu$L of 0.1 M HCl. The solutions were then analyzed by RP-HPLC.

**LTEN glutamyl *p*-cresol ester (11a):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.18 (d, $J$ = 8.6 Hz, 2H, *H*-ar), 6.98 (d, $J$ = 8.6 Hz, 2H, *H*-ar), 4.72 (t, 1H, α-Glu), 4.58 (dd, 1H, α-Asn), 4.41 (d, 1H, α-Thr), 4.16 (m, 1H, $\beta$-Thr), 3.99 (t, 1H, α-Leu), 2.78 (m, 2H, $\beta$-Asn), 2.69 (t, 2H, $\gamma$-Glu), 2.33 (s, 3H, C$H_3$−Ar), 2.29 (m, 1H, $\beta$-Glu), 2.02 (m, 1H, $\beta$-Glu), 1.70 (m, 3H, $\beta$-Leu + $\gamma$-Leu), 1.24 (d, 3H, C$H_3$−Thr), 0.95 (m, 6H, C$H_3$-Leu); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 174.92, 174.17, 173.33, 172.92, 171.87, 171.05, 150.02, 136.66, 130.81, 122.48, 68.42, 60.45, 53.47, 52.93, 50.69, 41.60, 37.64, 31.04, 28.36, 25.39, 23.16, 21.93, 20.84, 20.18; ES-MS $M_r$ 565.4, high-resolution FT-ICR MS 565.2827, calced for $C_{26}H_{39}N_5O_9$, 565.2748 (monoisotopic).

**LTEN glutamyl *p*-thiocresol ester (11b):** $^1$H NMR (300 MHz, $CD_3OD$, ppm) $\delta$ 7.34 (s, 4H, *H*-ar), 4.60 (m, 1H, α-Glu), 4.49 (m, 1H, α-Asn), 4.45 (m, 1H, α-Thr), 4.12 (m, 2H, α-Leu + $\beta$-Thr), 2.85 (m, 2H, $\beta$-Asn), 2.79 (m, 2H, $\gamma$-Glu), 2.38 (s, 3H, C$H_3$−Ar), 2.27 (m, 1H, $\beta$-Glu), 2.03 (m, 1H, $\beta$-Glu), 1.69 (m, 3H, $\beta$-Leu + $\gamma$-Leu), 1.23 (d, 3H, C$H_3$−Thr), 0.88 (m, 6H, C$H_3$−Leu); $^{13}$C NMR (75 MHz, $CD_3OD$, ppm) $\delta$ 203.03, 175.91, 172.91, 171.73, 171.29, 142.00, 135.62, 131.33, 123.95, 68.27, 60.11, 53.19, 52.79, 51.64, 40.94, 39.61, 37.80, 27.80, 24.87, 22.89, 22.01, 21.86, 19.85; ES-MS $M_r$ 581.1, calcd for $C_{26}H_{39}N_5O_8S$, 581.3 (monoisotopic).

**Hydrolysis of the Esters 11a and 11b.** The following buffers were used: (1) 0.1 M $NH_4HCO_3$ (pH 8); (2) 0.1 M $NaCO_3$/$NaHCO_3$ buffered to pH 9.2; (3) 0.1 M $NaCO_3$/$NaHCO_3$ buffered to pH 10. Batches of 500 $\mu$g of ester were dissolved in 500 $\mu$L of buffer. After the required time, the pH was adjusted to 2 using TFA and the solution was analyzed by LC/MS. For the peroxide-catalyzed experiments, hydrogen peroxide (0.3% in water) was added to the buffered solution.

**Human MRP14(89-114) (19).** Five hundred micrograms of the crude product (HF/*p*-cresol (9:1), 0 °C, 1 h) was dissolved in buffer A and purified by preparative RP-HPLC. ES-MS: $M_r$ 2728.7 (av); high-resolution FT-ICR MS 2727.3019 (monoisotopic), calcd for $C_{114}H_{171}N_{38}O_{39}S$, 2727.2208 (monoisotopic).

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***p*-Cresol Esters of MRP14(89-114) (20a−d).** The crude peptide (HF/*p*-cresol (9:1), 0 °C, 1 h) was dissolved in buffer A, and preparative RP-HPLC was performed. The separated *p*-cresol esters were then combined and lyophilized; yield 21% (w/w). ES-MS: $M_r$ 2818.8, calcd for $C_{121}H_{176}N_{38}O_{39}S$, 2819.0 (av).

***p*-Thiocresol Esters of MRP14(89-114).** The crude peptide (HF/*p*-thiocresol (9:1), 0 °C, 1 h) was dissolved in buffer A, and preparative RP-HPLC (C-18) was performed. The separated *p*-thiocresol esters were then combined and lyophilized; yield 29% (w/w). ES-MS: $M_r$ 2834.5, calcd for $C_{121}H_{176}N_{38}O_{38}S_2$, 2835.1 (av).

**Hydrolysis of *p*-Cresol Esters 20a−d.** Five hundred microgram batches of the MRP14(89-114) *p*-cresol esters were treated with the following solutions: (a) 500 $\mu$L of 0.1 M $NaHCO_3$ (pH 8.5) for 24 h at room temperature, (b) 500 $\mu$L of 0.1 M $NH_4HCO_3$ (pH 8.5) for 24 h at room temperature, (c) 500 $\mu$L of 0.1 M NaOH (pH 13) for 1 h at room temperature, and (d) 500 $\mu$L of 0.1 M $NaHCO_3$ (pH 8.5) containing 25 equiv of $H_2O_2$ for 5 min at room temperature. All reactions were monitored by LC/MS.

**Human MRP14(89-114) Glutamyl *p*-Cresol Ester, $NH_4HCO_3$ Treated.** Five hundred micrograms of the MRP14(89-114) cresol ester was then treated with 0.1 M $NH_4HCO_3$ (pH 8.5) and purified by RP-HPLC. ES-MS: $M_r$ 2727.2 (av); high-resolution FT-ICR MS, 2726.3033 (monoisotopic); calcd for $C_{114}H_{171}N_{39}O_{38}S$, 2726.2368 (monoisotopic).

**Human MRP14(89-114) Ketenebisthioacetals.** Five hundred micrograms of the crude product (HF/*p*-thiocresol (9:1), 0 °C, 1 h) was dissolved in buffer A and purified by RP-HPLC. ES-MS: $M_r$ 2940.3, calcd for $C_{128}H_{182}N_{38}O_{37}S_3$, 2941.3 (av).

**Human MRP14(89-114) Trithio Ortho Ester.** Five hundred micrograms of the crude product (HF/*p*-thiocresol (9:1), 0 °C, 1 h) was dissolved in buffer A and purified by preparative RP-HPLC. ES-MS: $M_r$ 3065.0, high-resolution FT-ICR MS, 3063.3052, calcd for $C_{135}H_{190}N_{38}O_{37}S_4$, 3063.3037 (monoisotopic).

**Reduction of MRP14(89-114)Met94[O] (19a).** One milligram of MRP14(89-114)Met94[O], **19a**, was dissolved in a solution containing 15% (v/v) acetic acid and 10% (v/v) *N*-mercaptoacetamide. The mixture was placed under a $N_2$ atmosphere and then incubated at 37 °C for 18 h. The conversion to **19** was quantitative as determined by RP-HPLC analysis. After isolation and lyophilization, the yield was found to be 80% (w/w).

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