Preparation and Biological Activity of Amino Acid and Peptide Conjugates of Antitumor Hydroxymethylacylfulvene

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The primary hydroxyl group in hydroxymethylacylfulvene, a potent antitumor drug, is readily replaced by thiols including cysteine, *N*-acetylcysteine, homocysteine, and glutathione. Best yields are obtained when reaction is carried out in the presence of dilute sulfuric acid. A variety of sulfur-containing analogues have been prepared, and their toxicity to tumor cells was examined.

Introduction

Hydroxymethylacylfulvene (**1**, HMAF, MGI 114; Chart 1)[1] is a promising antitumor compound derived from the sesquiterpene illudin S (**2**).[2] The latter is extremely toxic to animals but possesses a low therapeutic index when tested in tumor-bearing mice.[3] In contrast HMAF (**1**) produces complete tumor remission in a variety of xenograft models.[4] The compound is now undergoing phase II trials against several types of solid tumors.[5]
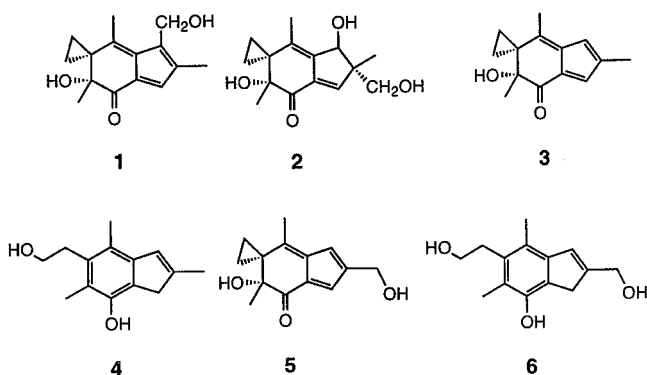
Studies of the mechanism of toxicity of illudins indicate that they behave as alkylating agents. Illudin S reacts spontaneously at room temperature with thiols, such as cysteine or glutathione (GSH), at an optimum pH of about 6, and toxicity to myeloid leukemia cells (HL60) can be modulated by altering glutathione levels in the cells.[6] The reaction of illudin S with GSH is illustrated in Scheme 1. A Michael-type addition to the α,β-unsaturated ketone gives a cyclohexadiene intermediate, an extremely reactive alkylating agent, which is converted rapidly to a stable aromatic product.

Illudin S undergoes enzymatic reduction with NADPH and rat liver cytosol giving a similar reactive intermediate and aromatic product (Scheme 1).[7] Thus, there are two ways in which alkylation involving the cyclopropane ring can be triggered.
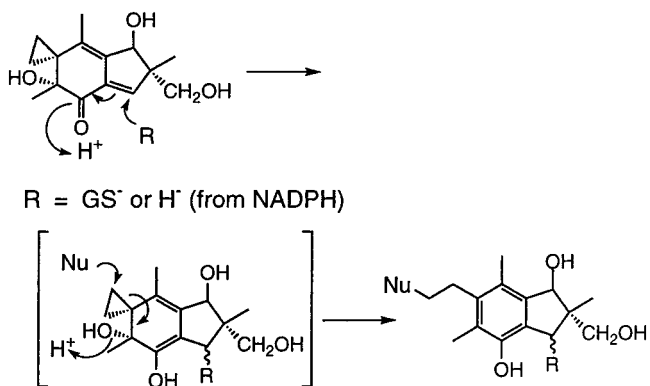
Acylfulvene (**3**), formed by treatment of illudin S with dilute sulfuric acid, undergoes similar reactions to illudin S but at a much slower rate.[8] The product from reaction with NADPH has the structure **4**. In addition, the enzymatic reaction gives hydroxylated products **5** and **6**.[9] Toxicity of acylfulvene (**3**) to HL60 cells is more than 2 orders of magnitude lower than that of illudin S. However, this reduced toxicity is accompanied by greatly improved selectivity in toxicity to malignant cells versus normal cells.[10]

Reaction of illudin S (**2**) with formaldehyde and sulfuric acid gives hydroxymethylacylfulvene (**1**).[1] Although this compound **1**, like acylfulvene (**3**), reacts slowly with thiols and with NADPH, it is substantially more toxic than **3**.[1] Most remarkable is the high

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Chart 1

Scheme 1

R = GS⁻ or H⁻ (from NADPH)

therapeutic index of **1** when tested on a variety of human tumors implanted in nude mice.[4] The primary allylic hydroxyl gives improved hydrophilicity which may be a factor. This group is extremely reactive to nucleophilic substitution, which is of course affected by the pH of the medium. At neutral pH the reaction rate is quite slow, but it increases as the concentration of the protonated hydroxyl form increases.[11] The reaction may contribute to the increased efficacy.
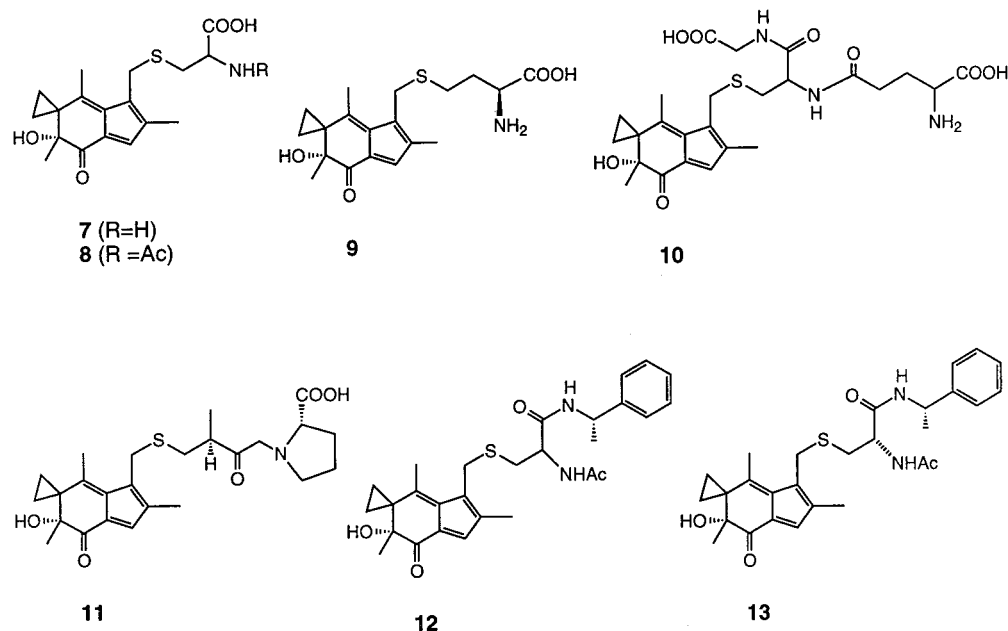
In this paper we report the preparation of conjugates of HMAF (**1**) with some biologically important thiols. In all cases the primary hydroxyl was displaced by thiol, leaving intact the cyclopropane and α,β-unsaturated ketone structures. Although the reactions could be effected simply by dissolving the thiol and HMAF (**1**)

Chart 2

in aqueous acetone, it was beneficial to add dilute sulfuric acid which increased the rate considerably.

Results and Discussion

Reaction of HMAF (**1**) with cysteine yielded compound **7** (Chart 2). A similar product **8** was obtained with *N*-acetylcysteine. Homocysteine reacted readily with HMAF (**1**) giving conjugate **9**. Likewise glutathione formed the expected product **10** with HMAF (**1**). Captopril, the angiotensin-converting enzyme (ACE) inhibitor,[12] reacted readily with **1** yielding product **11**. However, reaction of 6-mercaptopurine with HMAF (**1**) resulted in the opening of the cyclopropane, as evidenced (NMR) by disappearance of highfield multiplets (δ 0.7–1.5) for the cyclopropane protons and appearance of triplets (δ 3.0 and 3.5) for protons of a disubstituted ethane moiety. The product was not completely identified.

The thiol displacement reaction with HMAF (**1**) has been further employed to prepare other peptide conjugates. To check that the HMAF-cysteine conjugate would not be affected by the peptide-forming reaction, **8** was coupled to (*R*)-(+)-α-methylbenzylamine with *N*-hydroxybenzotriazole (HOBT) and *N,N*-diisopropylcarbodiimide (DIPC) in *N*-methylpyrrolidone (NMP). Products **12** and **13** were obtained as a mixture in the ratio 3:2. By using a different solvent (CH$_2$Cl$_2$), the racemization was decreased and the ratio of products was 6:1. HMAF-cysteine conjugate was then linked to various peptides constructed by solid-phase synthesis. The feasibility of the method was tested with three amino acids: Gly, Phe, and Leu. Thus FMOC-Gly was coupled to Rink acid resin (Novabiochem) using DIPC and (dimethylamino)pyridine in NMP. The yield of product was greater than 90%.

Removal of the FMOC protecting group with 20% piperidine in NMP, followed by coupling (with DIPC-HOBT) to a second FMOC-protected amino acid (Gly, Phe, or Leu), afforded 3 dipeptides. By repetition of this procedure, starting from FMOC-Phe and FMOC-Leu, a total of 9 dipeptides were obtained. Each of these was

Table 1. Peptide Conjugates of HMAF (**1**)[a]

X-LLL	X-LLF	X-LLG
X-LFL	X-LFF	X-LFG
X-LGL	X-LGF	X-LGG
X-FLL	X-FLF	X-FLG
X-FFL	X-FFF	X-FFG
X-FGL	X-FGF	X-FGG
X-GLL	X-GLF	X-GLG
X-GFL	X-GFF	X-GFG
X-GGL	X-GGF	X-GGG
X-LL	X-LF	X-LG
X-FL	X-FF	X-FG
X-GL	X-GF	X-GG

[a] X = HMAF (*N*-acetylcysteinyl); F = phenylalanine; L = leucine; and G = glycine.

coupled with Gly, Phe, and Leu yielding 27 tripeptides. The tripeptides, after removal of the FMOC group, were coupled in turn to HMAF-*N*-acetylcysteine. The solvent in this step was dichloromethane since NMP was found to cause extensive racemization of the chiral center in *N*-acetylcysteine. Cleavage of the peptides from the resin was effected with 10% acetic acid in dichloromethane.

HMAF-*N*-acetylcysteine was also coupled to 9 dipeptides and 3 amino acids giving a total of 39 peptide conjugates of HMAF (**1**). Overall, the yields were good to excellent. A list of dipeptide and tripeptide conjugates of HMAF-*N*-acetylcysteine is given in Table 1.

The method of solid-phase peptide synthesis proved to be an efficient way to link small peptides to HMAF, yielding a combinatorial library. Compounds were characterized by UV spectroscopy and in several cases by mass spectrometry.

Biological activity of several amino acid and peptide conjugates of HMAF (**1**) was determined using metastatic lung carcinoma (MV 522) cells. This cell line has been employed extensively in all our studies of toxicity and efficacy of illudin analogues.[11] The results, given in Table 2, show that the compounds were all less toxic than the parent HMAF (**1**). It is interesting to note that the glutathione conjugate (**10**) as well as the cysteine

Table 2. Toxicity (IC_{50}) Values for HMAF Analogues when Tested in MV 522 Cells

compd	IC_{50}[a]
1, Illudin S	4 ± 1 nM
2, HMAF	73 ± 8 nM
3, acylfulvene	350 ± 20 nM
7, HMAF-cysteine	29.2 ± 2.3 μM
8, HMAF-*N*-acetylcysteine	1.2 ± 0.32 μM
9, HMAF-homocysteine	>50 μM
10, HMAF-glutathione	15.5 ± 2.8 μM
11, HMAF-captopril	4.6 ± 0.25 μM
12, 8-α-methylbenzylamine	2.5 ± 0.12 μM
13, 8-α-methylbenzylamine isomer	NT
X-FGL (see Table 1)	37.2 ± 2.9 μM
X-LGF (see Table 1)	28.2 ± 1.4 μM
X-LLF (see Table 1)	>40 μM
X-LLL (see Table 1)	24.7 ± 3.9 μM

[a] For cytotoxicity tests, the compounds were dissolved in DMSO (1 mg/mL stock solution), and the solutions were diluted in 20% DMSO/phosphate-buffered saline just prior to addition to cultures of MV 522 cells. Control cells received equal amounts of the DMSO/phosphate-buffered saline. After incubation for 48 h, the cells were washed, trypan blue was added, and the cells were counted. These values correlate closely with those determined by colony-forming assay.

(**7**) and *N*-acetylcysteine (**8**) conjugates were substantially less toxic than HMAF (**1**). Conjugate **10** has been isolated from plasma of rats to which HMAF (**1**) was administered (iv).[13] This suggests that **10** was formed by enzymatic reaction (e.g. in the liver by GSH transferases), and HMAF (**1**) was thus partially detoxified. However, **10** still retains the α,β-unsaturated ketone and cyclopropylmethylcarbinol moieties which can react with nucleophiles. Therefore, **10** might still possess alkylating ability and hence antitumor activity. The same possibility holds for the other sulfur-containing analogues described above.

The lower toxicity of these analogues is possibly related to their larger size and polar functional groups which could impede their transport to sites where the acylfulvene moiety binds or reacts in a covalent manner. It is also possible that in vivo, the compounds might undergo degradation by peptidases thereby releasing the smaller and more reactive acylfulvene perhaps with enhanced efficacy.

We have demonstrated that it is feasible to prepare a variety of peptide conjugates of HMAF (**1**) using combinatorial chemistry methodology. It is possible to extend the method for preparation of further peptide conjugates. Certain peptides are known to bind to specific tumor-associated antigens in a manner analogous to the binding of antibodies to such antigens. It was reported recently that by coupling doxorubicin to two such peptides the efficacy of the drug against human cancer xenografts was enhanced.[14] Similar coupling experiments with HMAF (**1**) are now being investigated. In summary, this investigation has yielded many new analogues of a potent antitumor drug. One or more of these analogues, e.g. **7** and **10**, may be involved in metabolism of HMAF in the body. Also, some of these compounds may show greater efficacy than the parent HMAF thus leading to superior drugs.

Experimental Section

General. ^{1}H and ^{13}C NMR spectra were measured at 300 and 75 MHz. High-resolution mass spectra were determined at the University of Minnesota Mass Spectrometry Service Laboratory. Chromatography was done with silica gel (Davisil 230−425 mesh, Fisher Scientific, or Sorbsil, Phase Separations Ltd., U.K.), and solvents ethyl acetate and hexanes were used unless otherwise indicated. Analytical TLC was carried out on Whatman 4420 222 silica gel plates. Reactions were routinely monitored by TLC. Yields were calculated taking into account recovered starting materials.

Reaction of HMAF (1) with L-Cysteine. To a solution of HMAF (**1**; 118 mg, 0.48 mmol) in 1:1 1 M H_2SO_4:acetone was added L-(+)-cysteine (66.5 mg, 0.55 mmol). The mixture was stirred at room temperature for 24 h. The solution was extracted twice with CH_2Cl_2 to remove unreacted HMAF (**1**) and neutralized by adding sodium acetate. After removing the solvent, the crude product was chromatographed on a reversed-phase column (sorbsil) with $MeOH:H_2O$. A yield of 120 mg (72%) of product **7** was obtained as a yellow gum: ^{1}H NMR (CD_3OD) δ 0.78 (m, 1H), 0.89 (m, 1H), 1.06 (m, 1H), 1.31 (s, 3H), 1.43 (m, 1H), 2.15 (s, 3H), 2.21 (s, 3H), 2.91−4.02 (m, 8H), 7.04 (s, 1H); HRMS (FAB) for $C_{18}H_{23}O_4NS$ (M + H)$^+$ calcd 350.1420, found 350.1460.

Reaction of HMAF (1) with *N*-Acetylcysteine. To a solution of HMAF (**1**; 36 mg, 0.146 mmol) in 1:1 1 M H_2SO_4:acetone (3 mL) was added *N*-acetylcysteine (22.4 mg, 0.137 mmol) at room temperature. The mixture was stirred for 22 h and then was extracted with ethyl acetate. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, and the solution was dried over $MgSO_4$. After concentration, the crude product was chromatographed (davisil, 2−5% acetic acid added to the normal solvent mixture, ethyl acetate and hexanes). A yield of 445.5 mg (85%) product **8** was obtained. Compound **8** was a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.72 (m, 1H), 1.09 (m, 1H), 1.23 (m, 1H), 1.36 (s, 3H), 1.47 (m, 1H), 2.07 (s, 3H), 2.10 (s, 3H), 2.13 (s, 3H), 2.97 (m, 1H), 3.14 (m, 1H), 3.82 (dd, 3.82), 4.80 (m, 2H), 6.56 (d, *J* = 7.2 Hz), 7.10 (s, 1H); MS *m/z* 391 (M$^+$), 373, 229, 185; HRMS for $C_{20}H_{25}NO_5S$ calcd 391.1455, found 391.1452.

Reaction of HMAF (1) with DL-Homocysteine. To a stirred solution of HMAF (**1**; 100.7 mg, 0.41 mmol) in 1 M H_2SO_4 (1.5 mL) and acetone (1.5 mL) was added DL-homocysteine (55.4 mg, 0.41 mmol). The mixture was stirred at room temperature for 20 h and then it was partitioned between ethyl acetate and water. The aqueous phase was neutralized (NaHCO$_3$) and then concentrated under reduced pressure. The product **9** was isolated by reversed-phase chromatography (sorbsil, $MeOH:H_2O$) in a yield of 110 mg (75%): ^{1}H NMR (CD_3OD) δ 0.59 (m, 1H), 0.87 (m, 1H), 1.13 (s, 3H), 1.24 (m, 1H), 1.93 (s, 3H), 3.01 (s, 3H), 2.53 (t, 2H), 3.12 (s, 1H), 3.69 (m, 2H), 3.89 (br.s, 1H), 6.86 (s, 1H); MS *m/z* 364 (M + H)$^+$ 229; HRMS for $C_{19}H_{26}NO_4S$ calcd 364.1584, found 364.1580.

Reaction of HMAF (1) with Glutathione. To the solution of HMAF (**1**; 100 mg, 0.41 mmol) in 1:1 1 M H_2SO_4:acetone was added glutathione (reduced form, 120 mg, 0.39 mmol). The mixture was stirred at room temperature for 24 h. The solution was extracted twice with CH_2Cl_2 to remove unreacted HMAF (**1**) and the aqueous phase was neutralized by adding sodium acetate. The H_2O was removed under reduced pressure, and the crude product was chromatographed on a reversed-phase column (sorbsil) using water to flush out inorganic material and $MeOH:H_2O$ (1:20 to 1:5) to isolate the product **10** in a yield of 116 mg (56%): ^{1}H NMR (CD_3OD) δ 0.80 (m, 1H), 1.15 (m, 1H), 1.30 (s, 3H), 1.42 (m, 1H), 2.11 (s, 3H), 2.18 (s, 3H), 2.18 (m, 2H), 2.48 (m, 2H), 2.8 (m, 1H), 3.15 (m, 1H), 3.6 (m, 2H), 3.75 (m. 2H), 3.86 (m, 2H), 4.6 (m, 1H), 7.04 (s, 1H); HRMS (FAB) for $C_{25}H_{33}N_3O_8S$ (M + H)$^+$ calcd 536.2058, found 536.2045.

Reaction of HMAF (1) with Captopril [1-(3-Mercapto-2-methyl-1-oxopropyl)-L-proline]. To the solution of HMAF (**1**; 100 mg, 0.41 mmol) in 1 M H_2SO_4 (1.5 mL) and acetone (1 mL) was added captopril (88.3 mg, 0.41 mmol). The mixture was stirred at room temperature for 12 h then was partitioned between ethyl acetate and water. The organic layer was washed with NaHCO$_3$ solution and saline and then dried over MgSO$_4$. The product **11** was isolated by chromatography (davisil) in a yield of 153 mg (84%): ^{1}H NMR (CD_3OD) δ 0.70

(m, 1H), 105 (m, 1H), 1.195 (d, $J = 6.5$ Hz, 3H), 1.34 (s, 3H), 1.47 (m, 1H), 2.09 (s, 3H), 2.12 (s, 3H), 2.59 (m, 1H), 2.76 (m, 1H), 2.87 (m, 1H), 3.66 (s, 2H), 3.58 (m, 2H), 3.77 (s, 2H), 4.54 (m, 2H), 7.10 (s, H); HRMS (E1) for $C_{24}H_{31}O_5NS$ calcd 445.1915, found 445.1917.

Reaction of 8 with (+)-α-Methylbenzylamine. To a solution of **8** (40 mg, 0.102 mmol) in dichloromethane (1 mL) were added *N*-hydroxybenzotriazole (20 mg, 0.132 mmol), *N,N*-diisopropylcarbodiimide (20 μL, 0.12 mmol), and D-(+)-α-methylbenzylamine (12 μ L, 0.093 mmol). The mixture was stirred for 1.5 h at room temperature and then partitioned between EtOAc and water. The organic extract was dried over $MgSO_4$. After concentration, the crude product was chromatographed (davisil) to give 33.6 mg of compound **12** (73%) and 5.8 mg of **13** (13%).

Compound **12** was a yellow gum: ^1H NMR (CDCl$_3$) δ 0.70 (m, 1H), 1.07 (m, 1H), 1.29 (m, 1H), 1.35 (s, 3H), 1.48 (d, $J = 6.9$ Hz, 3H), 1.51 (m, 1H), 1.93 (s, 3H), 2.09 (s, 3H), 2.10 (s, 3H), 2.91 (m, 2H), 3.84 (dd, 2H), 4.61 (m, 1H), 5.03 (m, 1H), 6.64 (d, $J = 7.8$ Hz, 1H), 7.07 (s, 1H), 7.26 (m, H); ^{13}C NMR (CDCl$_3$) δ 197.5, 170.4, 169.2, 159.7, 142.6, 142.0, 138.3, 134.9, 129.8, 128.7, 127.4, 126.4, 125.9, 77.4, 52.5, 49.3, 37.7, 34.4, 29.8, 27.6, 23.1, 22.1, 16.3, 14.3, 13.1, 9.5; HRMS for $C_{28}H_{34}N_2O_4S$ calc: 494.2241, found 494.2225.

Compound **13** was a yellow gum: ^1H NMR (CDCl$_3$) δ 0.70 (m, 1H), 1.07 (m, 1H), 1.32 (m, 1H), 1.34 (s, 3H), 1.45 (m, 1H), 1.48 (d, $J = 6.9$ Hz, 3H), 2.03 (s, 3H), 2.05 (s, 6H), 2.76 (m, 1H), 2.87 (m, 1H), 3.73 (dd, 2H), 4.50 (m, 1H), 5.03 (m, 1H), 6.46 (d, $J = 7.5$ Hz, 1H), 6.77 (d, $J = 7.8$ Hz, 1H), 7.05 (s, 1H), 7.31 (m, 5H); HRMS for $C_{28}H_{34}N_2O_4S$ calcd 494.2241, found 494.2238.

Solid-Phase Synthesis of HMAF-*N*-acetylcysteine Linked to Phe, Gly, and Leu and to Combinations of Dipeptides and Tripeptides from These Amino Acids. Rink acid resin (purchased from Novabiochem) was used in the solid-phase reactions. The first *N*-(9-fluorenylmethoxycarbonyl)-protected amino acid was coupled to the resin via bond formation between its C-terminus and the hydroxyl group of the resin using DIPC (diisopropylcarbodiimide) and DMAP (4-(dimethylamino)pyridine). The percent yield was calculated after the coupling, and, if it was below 90%, the coupling reaction was repeated until the yield exceeded 90%. The *N*-(9-fluorenylmethoxycarbonyl) group (Fmoc group) of the first amino acid was removed by treatment with 20% piperidine in 1-methyl-2-pyrrolidinone (NMP). A Kaiser's test was performed to check the result of the deprotection. The three Kaiser's reagents (ninhydrin, phenol and potassium cyanide) were added to an aliquot of resin sample to yield a light yellowish solution. The mixture was heated at 100 °C for 3 min, and if the solution turned dark purple (positive result), the Fmoc group was considered to have been removed. The next amino acid was coupled by peptide bond formation using DIPC/HOBT (1-hydroxybenzotriazole). NMP was used as the solvent for deprotection, coupling, and washing, and dichloromethane was used as the drying reagent. Another Kaiser's test was then performed, with no color change (negative result), indicating that the coupling was successful. The coupling reaction was repeated to add additional amino acids. Compound **8** was coupled to the final resin-bound amino acid or peptide using dichloromethane as the solvent. NMP was not used as the solvent for the coupling reaction with compound **8** because NMP has been shown to cause inversion of the chiral center in cysteine, yielding a racemic mixture. Additionally, HOBT was not used in the coupling reaction with compound **8** because it may also contribute to the formation of a mixture of products. The cleavage of the resin from the peptide was achieved by treatment with 10% acetic acid in dichloromethane. The products were analyzed by UV and mass spectroscopy. HMAF gives two maximal UV absorbances at about 210 and 330 nm, and so the formation of products was confirmed by the presence of these two absorbances. Mass spectroscopy was also used to confirm the presence of the desired products. Spectral properties for selected peptides are as follows.

N-**AcetylCys-Phe-Gly-Leu:** $λ_{max}$ 330 nm ($ε$ 1240); HRMS (FAB) for $C_{37}H_{48}N_4O_8S$ calcd 708.3195, found 709.3298 (M + H)$^+$.

N-**AcetylCys-Leu-Gly-Phe:** $λ_{max}$ 330 nm ($ε$ 2220); HRMS (FAB) for $C_{37}H_{48}N_4O_8S$ calcd 708.3195, found 709.2211 (M + H)$^+$.

N-**AcetylCys-Leu-Leu-Phe:** $λ_{max}$ 330 nm ($ε$ 2060); HRMS (FAB) for $C_{41}H_{56}N_4O_8S$ calcd 764.3822, found 765.3941 (M + H)$^+$.

N-**AcetylCys-Leu-Leu-Leu:** $λ_{max}$ 330 nm ($ε$ 4600); HRMS (FAB) for $C_{38}H_{58}N_4O_8S$ calcd 730.3978, M$^+$ not observed.

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