J_{BX} = 8.1, J_{BA} = 17.5), 2.04 (s, 3 H, methyl). Second-order small splittings of about 1 Hz were seen also in the AB and methyl signals. The ABX pattern of pyrazoline **5a** has been reported.[4]

Anal. Calcd for $C_{32}H_{30}N_4$: C, 81.7; H, 6.42; N, 11.9. Found: C, 81.1; H, 6.38; N, 11.5.

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Convenient Synthesis of C-Terminal Peptide Analogues by Aminolysis of Oxime Resin-Linked Protected Peptides

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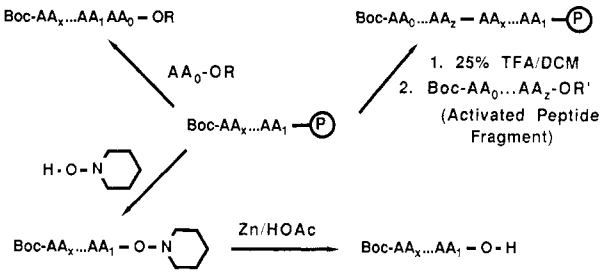
Methods for introducing C-terminal alkylamides into synthetic peptides have been either limited to special cases or restricted to peptides without ester side chain protecting groups. A structure activity research program required the preparation of a series of human epidermal growth factor (h-EGF) and renin inhibitor analogues with variable C-terminal functionalities. Peptides linked to *p*-nitrobenzophenone oxime resins are known to be displaced by amino acid esters or partially protected peptide fragments without disturbing side chain ester functionalities. We report now that the utility of oxime resins has been extended to the preparation of C-terminal alkylamides. Fully protected h-EGF (34–43)–*p*-nitrobenzophenone oxime resin was treated with ethylamine or cyclohexylamine in dichloromethane (DCM) for 1–5.5 h. The respective C-terminal amide products were produced in high purity. In addition, Boc-statine-Ile-oxime resin has been treated with 2-(aminomethyl)pyridine (AMP) in DCM for 2 h to give the respective Boc-statine-Ile-AMP in high purity. We conclude that the oxime resin offers a convenient and versatile method for the preparation of C-terminal peptide alkylamides. It also offers a convenient route for preparation of a wide variety of C-terminal analogues from a single synthesis.

Peptides systematically modified at the C-terminus with various alkylamide functionalities can be a useful addition to the biological evaluation of a peptide structural series. However, the methods for preparing such compounds are either severely limited or cumbersome. The (chloromethyl)polystyrene and [4-(methyloxy)phenyl](acetamidomethyl)polystyrene (1% divinylbenzene) (PAM) resins that are most commonly used for solid-phase peptide synthesis (SPPS) are not generally used for synthesis of peptides that contain C-terminal substituted amides.[1a,2] Benzhydrylamine and *p*-methylbenzhydrylamine resins have been developed primarily for the synthesis of unsubstituted C-terminal amides.[3] Recently a method for synthesizing *N*-methyl and *N*-ethyl carboxamide derivatives on polystyrene resins[4,5] has been reported, but its general utility, especially for hindered amines, is still unknown. Unfortunately this later approach suffers from the requirement that each desired carboxamide must be a separate synthesis on a separate resin. To date no general method has been recognized that will allow a wide variety of amines to displace on-resin peptides with ester side chain protecting groups or depsi backbone linkages.

One exception to the above generality might be met by polystyrene resins functionalized with an oxime group. Kaiser and co-workers[6,7] have explored the use of the *p*-nitrobenzophenone oxime resin in peptide synthesis and found that peptides attached to oxime resins could be displaced by nucleophilic agents (Scheme I). They found displacement could be effected by the C-α amines of amino acid esters under conditions mild enough to leave ester side chains unchanged and to allow the synthesis of depsi-

Scheme I. Displacement and Segment Condensation Options for Peptides on Oxime Resins



† Present address: Immunetech Pharmaceuticals, 1045 Roselle St., San Diego, CA 92122.

(1) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. (b) Abbreviations: HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; AMP, 2-(aminomethyl)pyridine; DMF, dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DTT, dithiothreitol; Et, ethyl; Chx, cyclohexyl; Boc, *tert*-butyloxycarbonyl; h-EGF, human epidermal growth factor; HPLC, high pressure liquid chromatography; Sta, statine, 4(*S*)-amino-3(*S*)-hydroxy-6-methyl-L-heptanoic acid.[14] Single letter amino acid codes: C, Cys, cysteine; E, Glu, glutamic acid; G, Gly, glycine; I, Ile, isoleucine; R, Arg, arginine; V, Val, valine; Y, Tyr, tyrosine.

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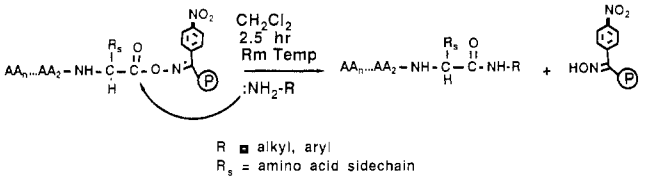
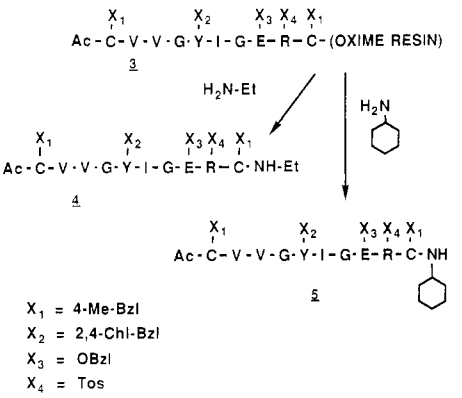
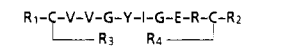
Scheme II. Aminolysis of Peptide from Oxime Resins



Scheme III. h-EGF-Oxime Resin Displacement by Ethylamine and Cyclohexylamine



peptides.[6-8] The methodology has also been used for segment condensation with C-terminal activated peptides directed to the free amino terminal of the resin-bound fragment[7a] and offers the potential for N-terminal amino group displacement with short protected peptides. Nucleophilic displacement of peptides from oxime resins appeared to be an attractive and general route to a wide variety of C-terminal alkylamides (Scheme II). Third loop analogues of EGF[1b] and transforming growth factor type α[9] have been reported to bind to the EGF receptor and may manifest interesting biological activity. To further explore these reports a series of h-EGF third loop analogs with different C-terminal alkylamides was needed for structure activity studies. The chosen h-EGF fragment amino acid sequence, **1a**, contains a glutamic acid residue



1a R_1 = H; R_2 = OH; R_3, R_4 = -S-S-

1b R_1 = Ac; R_2 = NH-Et; R_3, R_4 = -S-S-

1c R_1 = Ac; R_2 = NH-Chx; R_3, R_4 = -S-S-

2b R_1 = Ac; R_2 = NH-Et; R_3, R_4 = SH

2c R_1 = Ac; R_2 = NH-Chx; R_3, R_4 = SH

whose chain protecting group, usually a benzyl ester, represents a difficult case for aminolysis type reactions from the solid phase. Accordingly we prepared the fully protected h-EGF fragment, **3**, on an oxime resin to evaluate the synthesis of protected C-terminal analogues.

We report now our success in displacing the h-EGF third loop fragment, **3**, from an oxime resin at room temperature with ethylamine and cyclohexylamine. Ethylamine was chosen because **4** could be prepared conveniently by another route thereby allowing the oxime aminolysis procedure to be compared with other synthetic approaches. Cyclohexylamine was chosen because it is a more hindered amine than ethylamine. In addition, we report success in pilot studies wherein a peptide containing the bulky amino acid residue, isoleucine, attached to the oxime resin was efficiently displaced by AMP.

Table I. Procedure for Peptide Synthesis on Oxime Resins (1 mequiv)

step	reagents	time, min
(1) wash	DCM (3X)	1
(2) prewash	25% TFA/DCM (1X)	1
(3) deprotect	25% TFA/DCM (1X)	25
(4) wash	DCM (3X), *i*-PrOH (3X)	1
(5) wash	DCM (3X)	1
(6) neutralize	1% *N*-ethylmorpholine/DCM (2X)	0.5
(7) wash	DCM (1X)	0.5
(8) couple	3 mequiv of symmetrical anhydride[a]	*c*
(9) recouple if necessary		
(10) wash	(DCM (2X), *i*-PrOH (2X))2	1
(11) wash	DMF (2X)	1
(12) acetylate	capping mixture[b] (1X)	30
(13) recycle to step 1		

[a] Symmetric anhydride preparation: mix the Boc amino acid (6 mequiv) and DCC (3 mequiv) in DCM for 1 h, filter, concentrate in vacuo, and dissolve residue in minimal DMF. [b] Capping mixture: Ac_2O/DIEA/DMF (6:2:24 v/v/v). [c] Until ninhydrin negative.[10]

Standard procedures for SPPS had to be modified for the oxime resin (Table I) as the oxime to peptide ester linkage is more labile than the chloromethylated polystyrene or PAM resins to peptide linkages. To accommodate this lability the TFA deprotection step used 25% TFA in DCM instead of 50%. In addition the Boc deprotected and neutralized peptide was succeptable to cyclic eliminative loss from the resin. The potential chain loss required that the resin be capped at the end of each cycle to prevent truncated chain growth. Qualitative ninhydrin measurement was useful to estimate the extent of coupling and success of the capping reaction.[10] The chain loss might limit the usefulness of the oxime resin methodology if long peptides were to be made, but for the EGF decapeptides this did not present a problem. The final decapeptide on-resin yield was estimated to be 56% of the starting loading by quantitative Ellman's test.[3d]

The peptide displacement from the resin was carried out first with ethylamine and then with cyclohexylamine (Scheme III). The ethylamine aminolysis was completed without complications in 1 h. For cyclohexylamine, a more hindered amine, the displacement was complete in 5.5 h, but the reaction's minimum completion time was not determined. The final crude peptides showed few extraneous peaks on analytical HPLC. This result demonstrates the very high coupling yields/step achieved during solid-phase synthesis, the high quality of peptides that can be produced on oxime resins, and the ease and convenience with which the aminolysis can be carried out to produce a fully protected peptide.

The fully protected, crude peptide alkylamide was then subjected in order to HF cleavage (HF/*p*-cresol, 8:2, 0 °C, 1 h), extraction from the resin with 50% aqueous HOAc with 1% butanedithiol, precipitation with diethyl ether, trituration with diethyl ether and EtOAc, reduction with 0.1 M sodium phosphate and DTT and finally oxidation with diiodoethane in methanol.[11] The cysteines were ox-

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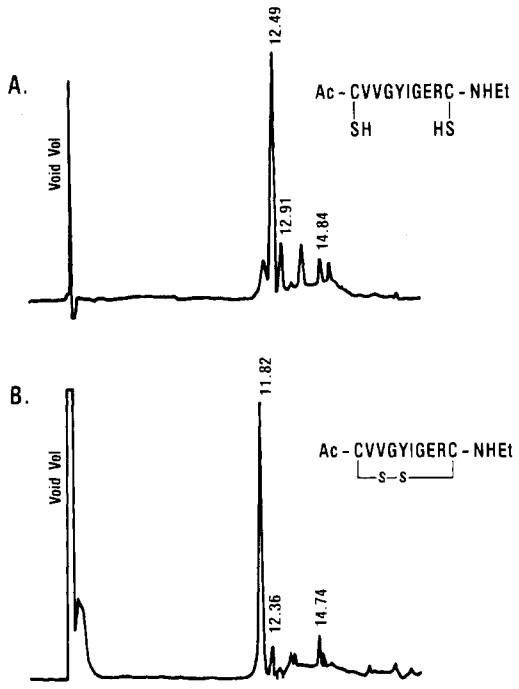
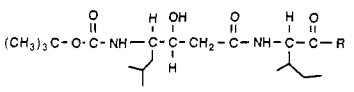


Figure 1. HPLC of crude HF product and oxidized crude HF product from the ethylamine c-terminal aminolysis of fully protected h-egf-oxime resine.

idized immediately after HF cleavage and before purification to minimize polymer formation and to maximize cyclized peptide. The diiodoethane oxidation method always produced a clean product.[11] The overall quality of the crude deprotected and oxidized ethylamide peptide was very high as shown by the HPLC (Figure 1). The cyclohexylamide was produced in similar high quality.

Initially one concern was that the alkylamine might also attack the h-EGF glutamic acid side chain protected with a benzyl ester. As there were no significant side products produced in this synthesis (judged by HPLC), the benzyl ester was stable, presumably, to the mild room temperatures and short exposure times used.

Finally, we were interested in evaluating the displacement reaction on a different peptide with a bulky C-terminal residue using a more complex amino nucleophile. For this model study we chose to evaluate the AMP displacement of a renin inhibitor precursor containing the unnatural transition state dipeptide mimic, statine, and the sterically hindered isoleucine attached to the oxime resin. Treatment of Boc-statine-Ile-oxime resin (**6a**) for 2 h with AMP in DCM afforded a single major product which was identical by HPLC with an authentic sample of **6b** produced by solution phase synthesis.[12] Similarly



6a R = Oxime Resin
6b R = AMP

Boc-Val-Ile-oxime resin treated with AMP produced a single major product. As with the ethyl- and cyclohexylamine displacements, the AMP reactions were clean and rapid.

(12) (a) Thaisrivongs, S.; Pals, D. T.; Kati, W. M.; Turner, S. R.; Tomasco, L. M.; Watt, W. *J. Med. Chem.* **1986**, *29*, 2080. (b) Thaisrivongs, S.; Pals, D. T.; Harris, D. W.; Kati, W. M.; Turner, S.R. *J. Med. Chem.* **1986**, *29*, 2088.

In summary we found that the oxime resin produced a good quality peptide, but the chain loss during each cycle may limit the procedure to smaller peptides. Nevertheless the convenience of resin preparation and the relatively standard nature of the coupling cycle make this an easily accessible method. The diversity of synthetic options afforded by this resin make it the method of choice for on-resin fragment condensations[7] and C-terminal modifications. It is particularly well suited for aminolysis reactions which produce a wide variety of C-terminal amide derivatives from a single precursor. To improve the utility of oxime resins for longer peptides we are planning to explore chemically modified oxime resins (other than *p*-nitrobenzyl substituted) for improved peptide chain stability during coupling cycles, and we plan to look at displacement by more hindered amines.

Experimental Section

All amino acids were purchased from Peptides International. All other reagents were purchased from Aldrich Chem. Co. or Sigma Chem. Co.

Analytical Systems. Only Burdick and Jackson HPLC solvents were used. Standard analytical HPLC conditions were as follows: Varian 6500 HPLC, Waters 490 monitor; column, Vydac 5u RP-C18, 25 cm × 0.3 cm; gradient; H_2O/CH_3CN (0.1% TFA) 100/0 to 20/80 over 20 min. Preparative HPLC was done on Lichroprep (E. Merck) or Vydac (The Separations Group) 20u RP-C18. Amino acid analyses were carried out with the following: Dionex Model D500; peptide hydrolysis conditions: constant boiling HCl/5% phenol, 110 °C/24 or 48 h.

***p*-Nitrobenzophenone Oxime Polystyrene Resin (Oxime Resin).** The ketone polymer was prepared from *p*-nitrobenzoyl chloride (0.08 mol) in nitro-benzene, anhydrous AlCl3 (0.11 mol), and Bio-Beads SX1 polystyrene (1% divinylbenzene copolymer, 80 g, BioRad, Inc.) in refluxing dichloroethane according to the procedure of Kaiser.[6] A KBr infrared spectrum of the dried, ground resin showed a carbonyl at 1665 cm^{-1} as well as a NO_2 band at 1527 cm^{-1} supporting the presence of *p*-nitrobenzoylated polystyrene (ketone resin).

The ketone resin was then refluxed with hydroxylamine hydrochloride (1.15 mol), pyridine and ethanol to give the oxime resin.[6] The carbonyl band at 1665 cm^{-1} disappeared over 15 h and a strong absorbance at 3506 cm^{-1} (NOH) appeared. Elemental analysis showed 2.4% nitrogen, which corresponds to a substitution level of 0.85 mmol of oxime/g of resin. The resin was sized and used without further characterization.

Boc-Cys(4MeBzl)-oxime Resin (Cys-P). Boc-Cys(4MeBzl) (1.3 mequiv) was in situ coupled with DCC (1.3 mequiv) and oxime resin (1 mequiv) in DCM for 20 h. After being washed with DCM and EtOH, the resin was capped with Ac_2O and DIEA in DMF to afford a final substitution of 0.5 mequiv/g.

Boc-Ile-oxime Resin (Leu-P). Boc-Ile-oxime resin (substitution level of 0.5 mmol/g), prepared analogously to that described for Cys-P, was deprotected and neutralized by the procedure shown in Table I.

Boc-Sta-Ile-oxime Resin. Boc-Ile-oxime resin (0.5 g, 0.5 mmol/g) was deprotected, neutralized, and then stirred for 2 h with a 2.5-fold excess of Boc-Sta preformed HOBt active ester (172 mg, 0.625 mmol of Boc-Sta; 84 mg, 0.625 mmol of HOBt; 128 mg, 0.625 mmol of DCC and 3 mL of DMF). The resin, which was ninhydrin negative, was thoroughly washed with DCM and EtOH and dried.

Boc-Sta-Ile-AMP (6b). Boc-Sta-Ile-oxime resin (**6a**), (100 mg) was treated with 50 mL of AMP in 10 mL of DCM for 2 h. The resin was filtered and washed with DCM and MeOH. The combined filtrates were concentrated in vacuo. The residue was dissolved in MeOH and injected on the analytical HPLC column (100/0 to 20/80 H_2O/CH_3CN + 0.1% TFA over 20 min, linear gradient). The major peak present at R_f 13.89 min was identical with that of an authentic sample of **6b**.

AcCys(4MeBzl)-Val-Val-Gly-Tyr(2,4Cl_2Bzl)-Ile-Gly-Glu-(OBzl)-Arg(Tos)-Cys(4MeBzl)-oxime Resin (3). The title peptide was elaborated according to the procedure described in Table I with single couplings for all amino acids except for arginine

and valine which required double couplings. The coupling progress was followed by the ninhydrin test.[10] The oxime resin was capped following each coupling cycle. Following the successful coupling of the final cysteine, it was deprotected, neutralized and acetylated.

Cyclic Disulfide AcCys-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys-NH-Et (1b). **3** (1 g) was stirred for 2.5 h with $EtNH_2$-saturated DCM. The cleaved peptide resin mixture was filtered, triturated in sequence with DCM, MeOH and TFA. The TFA-containing solution was concentrated to give 239 mg of crude protected peptide **4**. This material was subjected to HF/*p*-cresol (8:2) at 0 C for 1 h, concentrated in vacuo, taken up in 20 mL of 50% aqueous HOAc and 1% butanedithiol, and precipitated in diethyl ether (100 mL). The precipitate was washed sequentially with diethyl ether and EtOAc and taken up in 100 mL of 0.1 M sodium phosphate buffer (pH 8.0) and 20 mg of DTT to give **2b**. After 10 min ICH_2CH_2I in MeOH was added in aliquots[11a] until no free thiol remained (negative Ellman's test[13]). The reaction was quenched by the addition of glacial acetic acid. The crude peptide **1b** was purified on a RP18 column (gradient: 100/0 isocratic 30 min, 100/0 to 70/30 over 30 min, 70/30 to 60/40 over 180 min H_2O/CH_3CN + 0.1% TFA). The appropriate fractions were pooled, concentrated, redissolved in H_2O, and lyophilized. Anal. HPLC: one peak (R_f = 11.86 min, k' = 4.93). Amino Acid Anal.: Glu (1.03), Gly (2.08), Val (1.79), Ile (1.06), Tyr (1.09). FAB/MS: $(M + H)^+$ at *m/z* 1165 for mol wt 1164.

Cyclic Disulfide AcCys-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys-NH-Chx (1c). **3** (1.3 g) was treated with 1 mL of cyclohexylamine in DCM (50 mL) for 5.5 h at room temperature to give **5**. This peptide (294 mg crude), worked up as described for **4** above, was sequentially treated with HF to produce **2c**, oxidized to produce crude **1c**, and then purified on a RP18 column (gradient: 100/0 30 min, 100/0 to 60/40 over 30 min, 60/40 to 40/60 over 180 min H_2O/CH_3CN + 0.1% TFA). The peak tubes were pooled and rechromatographed in the same solvents (gradient 60/40 for 30 min, 60/40 to 40/60 over 240 min). The appropriate fractions were pooled, concentrated, redissolved in H_2O and lyophilized. Anal. HPLC: one peak (R_f = 13.45 min, k' = 7.41). Amino Acid Anal.: Glu (1.05); Gly (2.02); Cys/2 (0.88); Ile (1.03); Val (1.83); Tyr (1.2); Arg (1.11). FAB/MS: $(M + H)^+$ at *m/z* 1219 for mol wt 1218.

Acknowledgment. We thank J. Neilson for FAB/MS, Paul Meulman for infrared analysis, and Steve Turner and Suvit Thaisrivongs for the authentic sample of Boc-statine-Ile-AMP. We thank Professor E. T. Kaiser for reading the draft manuscript and making helpful comments.

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An Improved Synthesis of Plant Growth Regulating Steroid Brassinolide and Its Congeners

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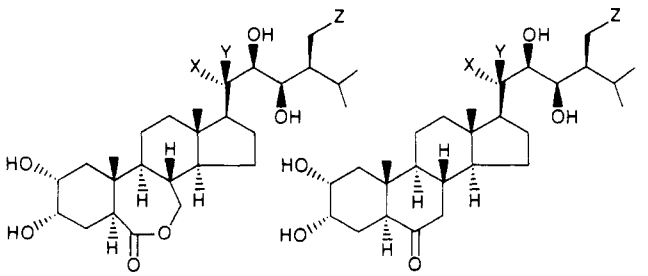
Brassinosteroids including brassinolide and castasterone have been synthesized by using pregnenolone as starting material.

Brassinolide (**1**), isolated from rape pollen (*Brassica napus* L.) is known to exhibit plant growth regulating activity. Because of its interesting physiological activity and its novel structural features, much effort has been devoted in recent years to develop the synthetic route to brassinolide and its analogues.[1]

Recently we succeeded in the stereoselective syntheses of brassinolide,[1] its enantiomer (22*S*,23*S*,24*R*)-22,23,24-epibrassinolide,[1] and 26,27-bisnorbrassinolide[2] by using a stereoselective reduction of the corresponding 5-ylidenetetronates as a key step to control the stereochemistry at the C-20, -22, -23, and -24 positions. In those syntheses, 6β-methoxy-3α,5-cyclopregnan-20-one was employed as a starting material, and the steroidal nucleus was manipulated after construction of the side chain. We here wish to report improved syntheses of brassinolide and its congeners including castasterone, where the steroidal nucleus was modified before construction of the side chain.

Chart I



1, brassinolide (X = Me, Y = Z = H)
2, 28-hydroxybrassinolide (X = Me, Y = H, Z = OH)
3, 20,28-dihydroxybrassinolide (X = Me, Y = Z = OH)
4, 20-hydroxybrassinolide (X = Me, Y = OH, Z = H)
5, 20-epibrassinolide (X = Z = H, Y = Me)
6, castasterone (X = Me, Y = Z = H)
7, 28-hydroxycastasterone (X = Me, Y = H, Z = OH)
8, 20,28-dihydroxycastasterone (X = Me, Y = Z = OH)
9, 20-hydroxycastasterone (X = Me, Y = OH, Z = H)
10, 20-epicastasterone (X = Z = H, Y = Me)

The structure–activity relationships of brassinosteroids have been investigated by several groups,[3] whose results

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