Simultaneous Multiple Synthesis of Peptide Amides by the Multipin Method. Application of Vapor-Phase Ammonolysis[1]

Andrew M. Bray,* Akhil G. Jhingran, Robert M. Valerio, and N. Joe Maeji

Chiron Mimotopes Pty. Ltd., 11 Duerdin Street, Clayton, Victoria 3168, Australia

Received July 29, 1993 (Revised Manuscript Received December 27, 1993)*

A method for simultaneously preparing large numbers of peptide amides is described. Side-chain deprotected, support-bound peptide esters **1** and **2** are incubated with ammonia/tetrahydrofuran vapor. The cleaved peptide amides **3** are then eluted from the support with a solvent of choice. The approach is demonstrated in conjunction with the multipin method of multiple peptide synthesis. In this study, chromophoric model systems of support-bound 4-(oxymethyl)benzamido esters of the genetically coded amino acids (except Cys) **4** and glycolamido esters of Ile, Val and Pro **5** were cleaved using the vapor from solutions of 30% ammonia in tetrahydrofuran, methanol, and 2-propanol. The best yields were obtained with 30% ammonia in tetrahydrofuran. When methanol was used as cosolvent, the amide products were contaminated with methyl ester. When ammonia gas alone was used, very poor yields were recorded. Although the hindered amino acid esters **4**(Ile, Val, Pro) cleaved with poor efficiency, the corresponding glycolamido esters **5**(Ile, Val, Pro) cleaved with >90% efficiency upon treatment with ammonia/tetrahydofuran vapor. Racemization studies on a selection of dipeptides cleaved by the method demonstrated that only low levels of racemate were generated. Four test peptides **16-19** were prepared and characterized to demonstrate the general utility of the method. The approach gives ready access to hundreds to thousands of discrete peptide amides in quantities (10-100 nmol) sufficient for most biological, immunological, and pharmacological studies.

Introduction

With increasing interest in peptidic drug candidates and synthetic vaccines, peptide bioscreening has become the focus of much research activity.[2] Simultaneous multiple peptide synthesis has, to a large extent, made this possible.[3] Access to large numbers of peptides can be achieved by either extensive parallel processing of discrete peptides[4-8] or by synthesizing peptide mixtures.[9-11] Preparing peptide mixtures simplifies the synthesis of very large numbers of peptides, but mixtures may not be suitable in all applications, hence, the need for synthetic methods capable of yielding thousands of discrete peptides in a relatively short time frame. However, many of the advantages of simultaneous synthesis are lost if peptides are side-chain deprotected, cleaved, and purified individually. For the full potential of simultaneous multiple synthesis to be realized, all synthetic steps should be performed in parallel. Following synthesis, peptides are further handled in preparation for biological assay. This can also present difficulties when very large numbers are encountered. If the end point of synthesis is a set of peptide solutions that can be used directly in a given bioassay, handling at this final stage is also simplified. By adopting these key measures, thousands of discrete peptides can be successfully prepared and screened within a relatively short time period.

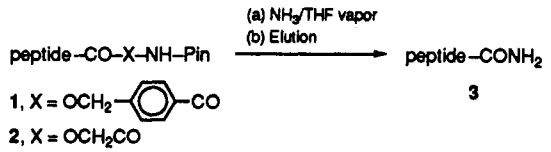
In constructing large peptide sets for bioscreening, strategies where cleavage and side-chain deprotection are distinct processes are preferable, as the support-bound peptides can be thoroughly washed prior to liberation. Peptide amides, however, are generally prepared by acidolytic cleavage of peptides from support-bound benzylamine- or benzhydrylamine-based handles.[12,13] As cleavage takes place in concert with side-chain deprotection, the liberated peptides require purification prior to biological screening. Although straightforward with small to medium numbers of peptides, the approach does not lend itself to the simultaneous production of hundreds to thousands of peptides. An alternative approach is the ammonolysis[14] of support-bound esters of side-chain deprotected peptides with liquid ammonia or ammonia/MeOH solution. Due to the need for individual handling, this procedure is also difficult to translate to situations where hundreds of discrete supports require attention. Well-sealed containers are required as trace amounts of moisture can result in the production of the acid product as a contaminant.[15] Furthermore, solution volatility demands low-temperature handling. Nevertheless, ammonolysis is an attractive procedure for mass cleavage if peptides are cleaved with ammonia vapor and then eluted from their supports with a solvent of choice.[1]

* Abstract published in *Advance ACS Abstracts*, February 15, 1994.

(1) Bray, A. M.; Maeji, N. J.; Jhingran, A. G.; Valerio, R. M. *Tetrahedron Lett.* **1991**, *32*, 6163-6166.
(2) Pavia, M. R.; Sawyer, T. K.; Moos, W. H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 387-398.
(3) Jung, G.; Beck-Sickinger, A. G. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 367-383.
(4) Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998-4002.
(5) Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5131-5135.
(6) Wolfe, H. R. Manual to RaMPS multiple peptide synthesis system, DuPont/NEN Co., Wilmington, DE, 1987.
(7) Frank, R.; Doring, R. *Tetrahedron* **1988**, *44*, 6031-6040.
(8) Maeji, N. J.; Bray, A. M.; Geysen, H. M., *J. Immunol. Methods* **1990**, *134*, 23-33.
(9) Geysen, H. M.; Rodda, S. J.; Mason, T. J. *Molecular Immunol.* **1986**, *23*, 709-715.
(10) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M; Knapp, R. J. *Nature* **1991**, *354*, 82-84.
(11) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84-86.
(12) Rink, H. *Tetrahedron Lett.* **1987**, *28*, 38-390.
(13) Bernatowicz, M. S.; Daniels, S. B.; Koster, H. *Tetrahedron Lett.* **1989**, *30*, 4645-4648.
(14) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 538-546.
(15) Brown, E.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc, Perkin Trans. 1* **1983**, 75-82.

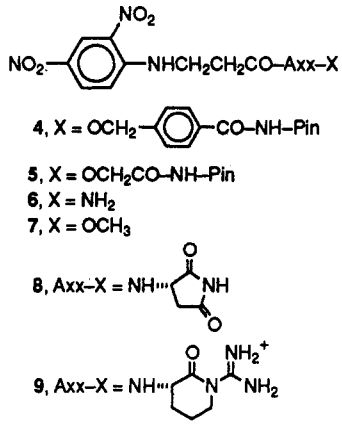
0022-3263/94/1959-2197$04.50/0

Scheme 1. Cleavage of Peptides with NH_3/THF Vapor[a]



[a] The cleaved peptides are eluted from the support with a solvent of choice.

In this study, ammonia vapor was used to cleave peptides from support-bound 4-(oxymethyl)benzamido esters[14] **1** and glycolamido esters[16] **2** as shown in Scheme 1. The approach was explored in conjunction with the multipin peptide synthesis method,[4] where peptide synthesis is performed on radiation-grafted polyethylene pins. Preliminary work[1] demonstrated that support-bound peptide esters **1** can be cleaved with ammonia vapor to yield the amide product **3**. Following cleavage, which is performed by storing the derivatized supports over a solution of ammonia in an organic solvent, the peptides are eluted from their respective supports. Either partially or wholly aqueous solutions can be used. By avoiding the need to carry out ammonolysis in solution, a technique well suited to parallel processing has been developed.

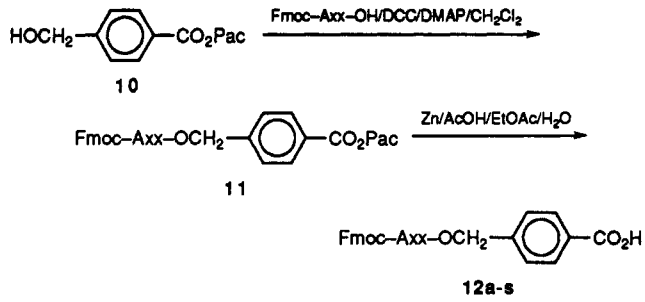


In a practical demonstration of the approach, a selection of peptides of biological interest were prepared and characterized. As the method is amenable to parallel processing, hundreds to thousands of discrete solution-phase peptides with amide C-termini can be prepared in a single calendar month. In the examples given here, each pin afforded a 150-μL solution containing at least 30 nmol of peptide. These quantities are adequate for large-scale screening in many biological, immunological, and pharmacological disciplines.[17,18]

Results

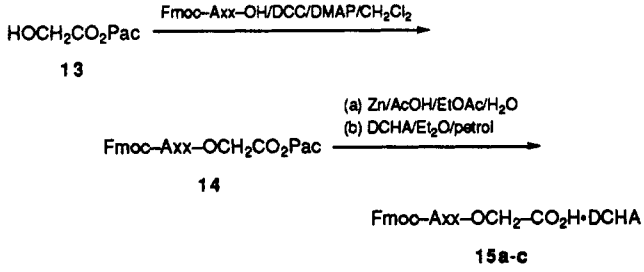
Preparation and Characterization of Ester Linkers. Peptide synthesis by the multipin method is performed on copolymer-grafted polyethylene pins which are arranged in an 8 × 12 format on a plastic holder. To easily accommodate the handling of several of these units at one time, coupling reactions and washes are performed in open containers under nonanhydrous conditions. To avoid the need to perform a moisture-sensitive ester bond-forming reaction directly on the pin surface, a series of protected amino acid esters **12a-s** containing the labile bond were prepared and coupled to the pins using DCC/HOBt. In this work, detachable pin heads[19] were used, enabling ready batchwise preparation of the functionalized pins. The compounds **12a-s** were protected for use with an Fmoc synthesis protocol. As shown in Scheme 2, Fmoc-protected amino acid was coupled to phenacyl ester **10**[20] by treatment with DCC in the presence of a catalytic amount of DMAP[21,22] to give the respective protected ester **11** in near-quantitative yield. The target compounds **12a-s** were liberated from their respective precursors (**11**) by treatment with zinc in AcOH. The solubility of **11** was improved in most instances by using ethyl acetate or DMF as a cosolvent, or by performing the reaction at 50 °C. Trituration of **12a-s** with ether/petrol effectively removed acetophenone, a byproduct of the deprotection, and all of the products except the Arg derivative, **12b**, crystallized. The His derivative, **12i**, was not obtained in an analytically pure form due to partial Trt loss during the deprotection step. The glycolate esters **15a-c** were prepared from **13**[20] (Scheme 3) and crystallized as their DCHA salts. Yields and characterization data are presented in Table 1.

Scheme 2. Preparation of Handles 12a-s[a]



[a] Axx = 19 of the genetically coded amino acids (Cys not included); protection as listed in Table 1.

Scheme 3. Preparation of Handles 15a-c[a]



[a] Axx = Ile, Pro and Val. See Table 1 for compound details.

Model Cleavage Studies. When dealing with thousands of support-bound peptides, the need to handle any group of peptides separately is a disadvantage. An optimal procedure should allow all peptides to cleave at one time under one set of conditions and with minimal side reactions. Hence, a series of model studies were under-

(16) Baleux, F.; Calas, B.; Mery, J. *Int. J. Pept. Protein Res.* **1986**, *28*, 22–28.
(17) Wang, J.-X.; Bray, A. M.; DiPasquale, A. J.; Maeji, N. J.; Geysen, H. M. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 447–450.
(18) Wang, J.-X.; DiPasquale, A. J.; Bray, A. M.; Maeji, N. J.; Geysen, H. M. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 451–456.
(19) Valerio. R. M.; Bray, A. M.; Campbell, R. A.; DiPasquale, A.; Margellis, C.; Rodda, S. J.; Geysen, H. M.; Maeji, N. J. *Int. J. Peptide Protein Res.* **1993**, *42*, 1–9.
(20) Bray, A. M.; Maeji, N. J.; Valerio, R. M.; Campbell, R. A.; Geysen, H. M. *J. Org. Chem.* **1991**, *56*, 6659–6666.
(21) Steglich, W.; Holfe, G. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 981.
(22) Gilon, C.; Kausner, Y.; Hassner, A. *Tetrahedron Lett.* **1979**, 3811–3814.

Table 1. Preparation and Characterization of Fmoc-Axx-$OCH_2C_6H_4CO_2H$ (12a-s) and Fmoc-Axx-OCH_2CO_2H·DCHA (15a-c)

	Axx	overall yield (%)	mp (°C)	$[\alpha]^{22}_D$ (c 1, DMF)	molecular formula	found (calcd) C	H	N
12a	Ala	68	142–145	−28.4	$C_{25}H_{23}NO_6$	70.19 (70.10)	5.21 5.20	3.17 3.41)
12b	Arg(Pmc)	61	91–95	−5.6	$C_{43}H_{46}N_4O_9S·CH_3OH$	63.87 (63.70	6.43 6.32	6.41 6.76)
12c	Asn(Trt)	82	125–128	−7.4	$C_{46}H_{38}N_2O_7$	75.40 (75.60	5.36 5.24	3.76 3.83)
12d	Asp(O^{t}Bu)	74	153–155	−23.0	$C_{31}H_{31}NO_8·0.5H_2O$	67.55 (67.13	5.82 5.82	2.29 2.53)
12e	Gln(Trt)	90	94–96	−9.0	$C_{46}H_{40}N_2O_7$	75.63 (75.79	5.69 5.41	3.43 3.76)
12f	Glu(O^{t}Bu)	77	149–150	−16.4	$C_{32}H_{34}NO_8·0.5H_2O$	67.80 (67.47	5.86 6.19	2.23 2.45)
12g	Gly	81	178–180	–	$C_{25}H_{21}NO_6·0.5H_2O$	68.04 (68.17	5.09 5.04	3.56 3.18)
12h	His(Trt)	88	132–134	−19.1	$C_{48}H_{39}N_3O_6$	69.26 (76.47	5.61 5.22	6.82 5.58)
12i	Ile	60	128–130	−18.6	$C_{29}H_{29}NO_6$	71.50 (71.44	6.13 6.00	2.71 2.87)
12j	Leu	92	172–178	−19.2	$C_{29}H_{29}NO_6·CH_3OH$	69.32 (69.35	6.03 6.40	2.80 2.70)
12k	Lys(Boc)	80	99–102	−13.1	$C_{34}H_{38}N_2O_8$	67.58 (67.76	6.62 6.36	4.66 4.65)
12l	Met	77	161–162	−24.4	$C_{28}H_{27}NO_6S$	66.58 (66.52	5.52 5.38	2.90 2.77)
12m	Phe	75	170–172	−20.9	$C_{32}H_{27}NO_6$	73.69 (73.69	5.22 5.19	2.69 2.60)
12n	Pro	65	164–166	−42.7	$C_{28}H_{25}NO_6·H_2O$	68.53 (68.70	5.40 5.56	3.14 2.86)
12o	Ser(^{t}Bu)	92	153–154	−1.6	$C_{30}H_{31}NO_7·H_2O$	67.39 (67.27	5.91 6.21	2.46 2.62)
12p	Thr(^{t}Bu)	79	142–144	+4.3	$C_{31}H_{33}NO_7$	70.18 (70.04	6.42 6.26	2.54 2.63)
12q	Tyr(^{t}Bu)	90	132–134	−17.1	$C_{36}H_{35}NO_7$	72.69 (72.83	6.18 5.94	2.74 2.36)
12r	Trp	68	144–149	−6.7	$C_{34}H_{28}N_2O_6$	73.07 (72.84	5.20 5.03	5.11 5.00)
12s	Val	70	133–137	−20.6	$C_{28}H_{27}NO_6$	71.30 (71.02	5.83 5.75	2.87 2.96)
15a	Ile	97	134–135	−16.1[a]	$C_{35}H_{48}N_2O_6$	70.86 (70.91	8.17 8.16	4.62 4.73)
15b	Pro	91	145–146	−48.3[a]	$C_{34}H_{46}N_2O_6$	70.77 (70.80	7.57 7.69	4.79 4.86)
15c	Val	57	133–134	−19.5[a]	$C_{34}H_{44}N_2O_6$	70.50 (70.56	8.16 8.01	4.73 4.84)

[a] c 1, MeOH.

taken to identify any C-terminal residues which could pose problems during large-scale handling. Cleavage studies were performed on the chromophoric systems 4 and 5, and cleaved peptide solutions were quantitated by determining their absorbances at 405 nm on a microtiter plate reader. Following trial cleavage, the remaining color was removed from the pin heads by treatment with 0.25 M NaOH in EtOH–water (1:1, 160 μL/pin). These solutions were also quantitated and were taken to represent 100% cleavage.

A series of ammonia vapor cleavage studies are summarized in Table 2. Three organic solvent-based ammonia solutions were used as the vapor source: THF, MeOH, and ^{i}PrOH. Pins derivatized with model systems 4 and 5 were side chain deprotected and then suspended over a 30% solution of ammonia for 24 h. Each pin was then eluted with EtOH/water (1:1) under the action of sonication. Of the three methods, that using NH_3/THF was generally the most efficient; many of the model systems afforded their respective amides (6) with greater than 90% efficiency. Notable exceptions were the sterically hindered systems 4(Ile, Pro, Val), which gave yields of 4%, 16%, and 7%, respectively. The glycolamido ester analogs 5(Ile, Pro, Val), however, gave excellent yields under these conditions. As 5(Pro) is less stable than 4(Pro),[20] the stability of 5(Ile, Val) to peptide synthesis was assessed. Hence, eight derivatized pins of each type were subjected to 12 coupling/deprotection cycles. Comparison of these pins with those receiving no treatment demonstrated that less than 5% (the limit of reliable detection) of the model systems were lost. The moderately hindered benzylic systems 4(Leu, Trp) cleaved with efficiencies of 61% and 67%, respectively, prompting an assessment of their corresponding glycolamido esters. However, 5(Trp) was found to be too labile for use in routine synthesis, with 70% loss over 12 coupling/deprotection cycles. Consequently, 5(Leu) was not assessed.

A majority of the amide products prepared by the NH_3/THF method were found to be homogeneous by HPLC. An exception was 6(Asn), which contained up to 20% of the imide[23] 8. Nevertheless, MS (positive ion CI-CH_4) demonstrated that the desired target was obtained, with an $[M + H]^+$ signal being observed at m/z 369. Analysis of 6(Arg) by MS confirmed that the lactam[24] 9 had not formed, with the required $[M + H]^+$ signal being observed

(23) Tesser, G. I.; Buis, J. T. W.A.R.M.; Wolters, E. T. M.; Bothe-Helmes, E. G. A.M. *Tetrahedron* **1976**, *32*, 1069–102.
(24) Bodanszky, M.; Martinez, J. *Synthesis* **1981**, 333–356.

Table 2. Model Cleavage Studies on Dnp-βAla-Axx-X-NH-Pin 4 (Axx) (X = $OCH_2C_6H_4CO$) and 5 (Axx) (X = OCH_2CO)

		cleavage efficiency (%)		
	Axx	NH3/THF vapor	NH3/^{i}PrOH vapor	NH3/MeOH vapor[a]
4	Ala	93	58	95 (73:17)
	Arg	96	70	96 (87:13)
	Asn	94[b]	96[b]	92 (100:0)[b]
	Asp	74	65	45 (74:26)
	Gln	90	55	95 (78:22)
	Glu	82	64	43 (75:25)
	Gly	95	94	95 (100:0)
	His	96	79	95 (91:9)
	Ile	4	2	29 (22:78)
	Leu	61	26	92 (55:45)
	Lys	94	67	97 (ND)
	Met	87	55	95 (74:26)
	Phe	91	57	96 (82:18)
	Pro	16	7	58 (34:66)
	Ser	95	89	93 (95:5)
	Thr	93	77	93 (89:11)
	Trp	67	22	96 (79:21)
	Tyr	73	41	91 (72:28)
	Val	7	3	31 (23:77)
5	Ile	89		
	Pro	94		
	Val	97		

[a] Amide/methyl ester ratio given in parentheses. [b] Product contains ca. 20% imide 8. ND, Not determined.

at m/z 411. Although Met oxidation has been found in peptides cleaved by the method[1,17] (including 18, see below), MS on 6(Met) only detected the reduced species with $[M + H]^+$ at m/z 386.

Most of the products generated in the NH_3/MeOH study were found to contain methyl ester 7, as demonstrated by HPLC. This result was confirmed by MS (positive ion CI-CH_4). For example, the 6(Ile) preparation gave a signal at m/z 383 in addition to the expected $[M + H]^+$ signal at m/z 368. Methyl esters 7 were the major products obtained from the NH_3/MeOH cleavage of 4(Ile, Pro, Val). As the cleavage of Ile and Val esters from resin with ammonia/MeOH can yield products contaminated with methyl esters,[25] the solution-phase ammonolysis of pins was assessed. When 4(Ile) was treated with a solution of 30% NH_3/MeOH for 24 h at 20 °C, 77% cleavage was achieved, with a 21:79 ratio of amide 6/ester 7 being produced. The observed amide/ester ratios obtained for solution-phase cleavage of 4(Pro, Val) were 72:28 and 39:61, respectively, with cleavage efficiencies on the order of 90% being achieved. Hence, the problem of ester contamination is not peculiar to the gas-phase cleavage method. When NH_3/^{i}PrOH was used as the NH_3 vapor source ester products were not observed. Yields, however, were inferior to those obtained with NH_3/THF. Interestingly, when liquid ammonia was used as the ammonia vapor source, cleavage efficiency was very poor (data not shown). Similarly, poor results were obtained when cleavage was attempted in a continuous stream of ammonia gas for 10 h (data not shown).

Racemization Studies. A series of L-Leu-L-Axx-NH_2 and D-Leu-L-Axx-NH_2 dipeptides[26] were prepared to assess the levels of racemization inherent in the NH_3/THF vapor cleavage method. The results of this study are presented in Table 3. As both diastereomers were prepared, the identification of the respective racemization products, L-Leu-D-Axx-NH_2 and D-Leu-D-Axx-NH_2, was straightforward by HPLC. Although peak overlap precluded assessment for some species, low racemization levels were generally found. To ensure adequate adsorption to the C-18 surface, the study was performed with side-chain protection in place. The highest degree of racemization was found in the case of L-Leu-Asp(O^{t}Bu)-NH_2. Nevertheless, the reported[27] high levels of DMAP-promoted racemization were not observed.

Table 3. Racemization Study on L-Leu-L-Axx-NH_2 and D-Leu-L-Axx-NH_2. Cleavage with NH_3/THF Vapor

dipeptide	t_R[a] (min)	% L-L or D-D[b]	% L-D or D-L[b]
L-Leu-L-Ala-NH_2	13.58	>99.9	<0.01
D-Leu-L-Ala-NH_2	13.18	0.2	99.8
L-Leu-L-Asp(O^{t}Bu)-NH_2	17.93	98.2	1.8
D-Leu-L-Asp(O^{t}Bu)-NH_2	16.85	1.6	98.4
L-Leu-L-Glu(O^{t}Bu)-NH_2	18.36	99.6	0.4
D-Leu-L-Glu(O^{t}Bu)-NH_2	18.03	0.5	99.5
L-Leu-L-Phe-NH_2	17.31	99.5	0.5
D-Leu-L-Phe-NH_2	17.05	0.5	99.5
L-Leu-L-Ile-NH_2	17.07	>99	ND
D-Leu-L-Ile-NH_2	16.28	0.5	99.5
L-Leu-L-Leu-NH_2	17.25	99.8	0.2
D-Leu-L-Leu-NH_2	16.79	0.2	99.8
L-Leu-L-Lys(Boc)-NH_2	18.43	99.9	0.1
D-Leu-L-Lys(Boc)-NH_2	18.22	0.4	99.6
L-Leu-L-Ser(^{t}Bu)-NH_2	17.24	99.2	0.8
D-Leu-L-Ser(^{t}Bu)-NH_2	16.98	1.1	98.9
L-Leu-L-Trp-NH_2	16.79	99.7	0.3
D-Leu-L-Trp-NH_2	17.18	0.2	99.8
L-Leu-L-Tyr(^{t}Bu)-NH_2	19.63	99.7	0.3
D-Leu-L-Tyr(^{t}Bu)-NH_2	19.49	<0.01	>99.9

[a] HPLC conditions given in the Experimental Section. [b] By peak areas. ND, not determined.

Application in Peptide Synthesis. Table 4 presents data for four peptides, 16–19, prepared to demonstrate the utility of the cleavage methodology. These peptides were assembled by an Fmoc synthesis protocol, together with ca. 3000 other peptides. Most coupling reactions were performed using DCC/HOBt in DMF. The sterically demanding Fmoc-Arg(Pmc)-OH and Fmoc-His(Boc)-OH were introduced using PyBOP[28] as the coupling reagent. Following side-chain deprotection, the support-bound peptides were sonicated in 0.1% HCl in aqueous MeOH and then soaked in ethanol to remove noncovalent impurities. Cleavage was effected with NH_3/THF vapor. The cleaved peptide amides were eluted from the pin surface into aqueous MeCN.

The test peptides were characterized by analytical reversed-phase HPLC, amino acid analysis, and MS. Amino acid ratios for the test peptides are presented in Table 5. Apart from Trp, which was not quantitated, the determined ratios were in good agreement with those anticipated. With extended hydrolysis times acceptable ratios were found for the relatively stable Val-Val moiety[29] within peptide 19. The amount of each peptide eluted from a single pin is given in Table 4. Analytical HPLC showed that the products were of reasonable purity; chromatograms of the unpurified products are presented in Figure 1. Peptides 16 and 17 were examined by positive-

(25) Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis, A Practical Approach*; IRL Press at Oxford Press: Oxford, 1989; pp 149–153.

(26) Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 6147–6150.

(27) Dhaon, M. K.; Olsen, R. K.; Ramasamy, K. *J. Org. Chem.* **1982**, *47*, 1962–1965.

(28) Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205–208.

(29) Grant, G. A. In *Synthetic Peptides: A User's Guide*; Grant, G.A., Ed., W. H. Freeman and Company: New York, 1992; p 217.

Table 4. Peptides Used in Method Appraisal (*16–19*)

	sequence	amt cleaved (nmol)	MS data (*m/z*) obsd signals	expected $[M + H]^+$	notes
16	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	65	1183, $[M + H]^+$	1183.4	LH-RH
17	Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH_2	42	1423, $[M + H]^+$	1423.9	granuliberin R
18	pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	29	2115.0, $[M + O + H]^+$ 1057.5, $[M + O + 2H]^{2+}$	2099.2	gastrin I (Human)
19	Thr-His-Gly-Ile-Arg-Pro-Val-Val-Ser-Thr-Gln-Leu-Leu-Leu-Asn-Gly-Ser-Leu-Ala-Glu-NH_2	36	2106.5, $[M + H]^+$ 1053.0, $[M + 2H]^{2+}$	2105.6	HIV envelope protein gp120 255-274

Table 5. Amino Acid Analysis of Peptides *16-19*[a]

	16	*17*	*18*	*19*
Ala		1.0 (1)	1.0 (1)	1.1 (1)
Arg	1.0 (1)	2.0 (2)		0.6 (1)
Asp[b]			1.1 (1)	1.1 (1)
Glu[c]	1.2 (1)		6.0 (6)	2.3 (2)
Gly	2.2 (2)	1.1 (1)	2.1 (2)	2.2 (2)
His	1.0 (1)			0.9 (1)
Ile		0.9 (1)		0.7 (1)
Leu	0.9 (1)	1.0 (1)	0.9 (1)	4.3 (4)
Met			1.1 (1)	
Phe		1.9 (2)	1.0 (1)	
Pro	1.0 (1)	2.1 (2)	1.0 (1)	1.1 (1)
Ser	0.9 (1)	1.0 (1)		2.1 (2)
Thr				2.0 (2)
Trp[d]	obsd		obsd	
Tyr	1.0 (1)	0.9 (1)	0.8 (1)	
Val				1.9 (2)[e]

[a] Values in parentheses are expected values. [b] Asn analyses as Asp. [c] Gln analyses as Glu. [d] Trp not quantitated. [e] 48 h hydrolysis.

ion FAB MS, while ion-spray MS was used in the case of the high molecular weight peptides **18** and **19**. The data are included in Table 4. The expected $[M + H]^+$ signals were observed for **16**, **17**, and **19**. MS confirmed that the base-sensitive sequence –Asn-Gly–[30] within **19** had not cyclized. The Met-containing peptide **18** was oxidized as evident by the [M + H + O]+ signal.

Discussion

When used in conjunction with the multipin method, the vapor-phase ammonolysis strategy allows rapid access to large sets of peptide amides which are suitable for bioscreening projects. Systematic cleavage studies on the chromophoric model systems **4** and **5** demonstrated that ammonolysis efficiency was generally high. However, the choice of solvent used in the ammonia vapor source solution was shown to be critical. Of the solutions examined, NH_3/THF was found to give the highest yields, while NH_3/MeOH afforded amide products **6** that were, in most instances, contaminated with the analogous methyl ester **7**. Very low yields were obtained when NH_3 alone was used. Although the sterically hindered systems **4**(Ile, Pro, Val) cleaved with low efficiency, high yields were obtained with the glycolamido ester analogs **5**(Ile, Pro, Val). Hence, esters of 19 of the genetically coded amino acids (Cys was not included in this study) can be cleaved together, as required by a multiple synthesis strategy.

An interesting result to emerge from this study is that ammonia vapor alone is inadequate for cleavage; a volatile cosolvent is required to assist in the solvation of the polymer matrix. With the dimethylacrylamide/methacrylic acid-based polymer used in this study, THF was found to be the most effective cosolvent. Due to the large amounts of methyl ester formed during cleavage, NH_3/MeOH was found to be an unsuitable ammonia source. Although ester formation was not encountered with NH_3/

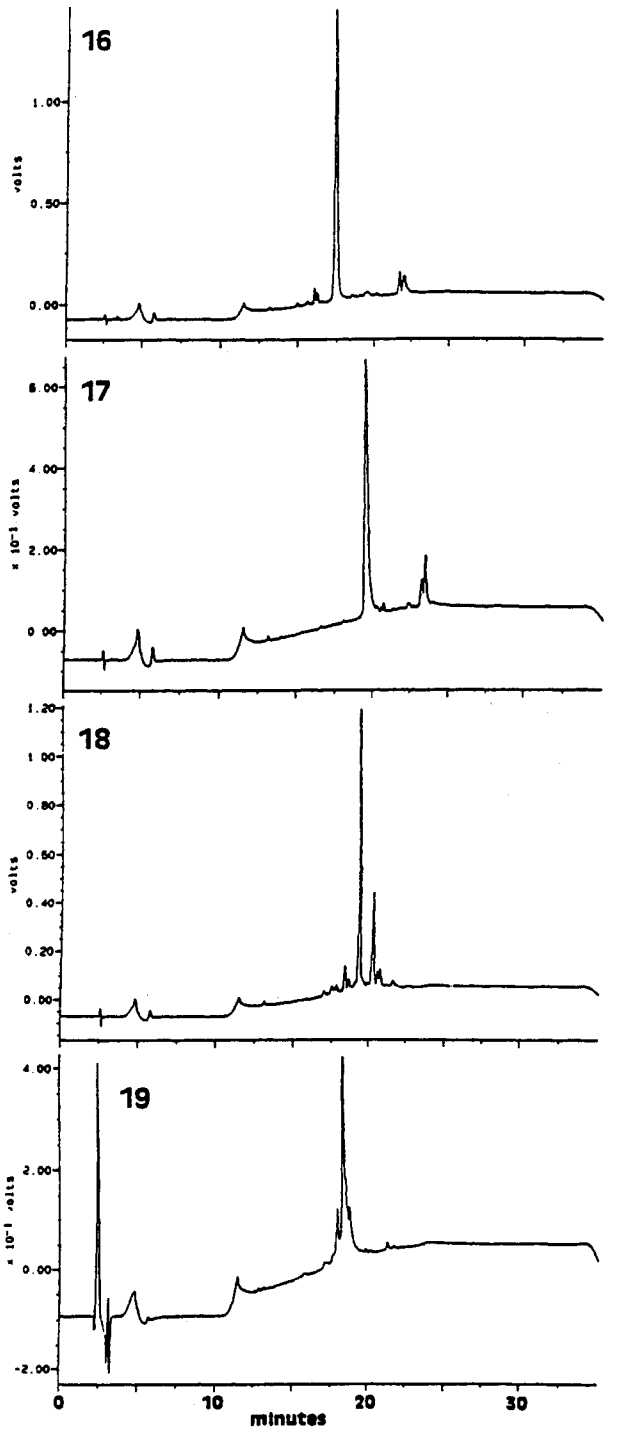


Figure 1. HPLC chromatograms of four test peptides cleaved with NH_3/THF vapor and eluted from the pin support with MeCN/H_2O (40:60). Detection at 214 nm. HPLC conditions are given in the Experimental Section.

(30) Capasso, S.; Mazzarella, L.; Zagari, A. *Peptide Res.* **1991**, *4*, 234–238.

^{i}PrOH, cleavage efficiencies were generally inferior to those obtained with NH_3/THF. It seems likely that other polymer matrices used for peptide synthesis may require other solvent compositions for optimal performance.

In this study, cleaved peptide amides were eluted from the support with aqueous-based solvent mixtures. This is possible as the peptides were prepared on a hydrophilic polymer matrix. Elution efficiency is greatly enhanced when performed under the influence of high power sonication. Even moderately insoluble peptides can be eluted from the support, although they may subsequently precipitate. In cases where small, hydrophilic peptides have been prepared, it is feasible to perform the elution with a wholly aqueous solution, such as physiologically compatible buffer. This is an advantage in large-scale bioscreening as postsynthesis peptide handling can be kept to a minimum. In general, however, a cosolvent such as acetonitrile would be added to assist peptide solubilization. This does not preclude the direct use of these solutions in bioassays as acetonitrile can be tolerated in some cell cultures.[31] Alternatively, peptide solutions can be lyophilized and reconstituted in an alternative solution.

A procedure suitable for mass peptide preparation must give minimal side reactions, and so assessment of potential side reactions formed an important component of this study. Esters of Asn, and to a lesser extent Gln, are prone to base-catalyzed imide formation.[23] The C-terminal Asn model peptide was found to contain 20% imide when cleaved with ammonia/THF vapor. In the case of Gln, the imide product was not observed. Neither was the related cyclization of -Asn-Gly- to the imide[30] encountered in the test peptide **19**. Although Arg esters are prone to base-catalyzed lactam formation,[24] this reaction was not observed either. The method was also shown to cause very little racemization. Hence, all 19 C-terminal types studied yielded the correct termini; only Asn gave a significant byproduct. Despite the observation that the model peptide 6(Met) was in the reduced form, the Met-containing peptide **18** was found to be oxidized. Generally, Met-containing peptides prepared by this method have been found to be partially or fully oxidized. Cys oxidation was not explored in this study but is known to be promoted by basic conditions if oxygen is not rigorously excluded. It should be noted that the issue of Met and Cys oxidation arises whenever peptides are handled in near neutral buffers typically used in bioscreening.

The simplicity of the technique not only lends itself to multiple synthesis but also to application in biological laboratories, where most bioscreening studies are undertaken. Provided an adequate NH_3/THF atmosphere is established, any number of discrete supports can be simultaneously cleaved. As the setup time is very short, and the procedure is performed in a sealed vessel, the supports and ammonia source solution can be protected from atmospheric moisture, hence minimizing side reactions. When applied to the multipin synthesis strategy, where peptides are prepared on organized modular supports, very large numbers of peptides can be readily handled. For example, 1440 peptides have been simultaneously cleaved by this method in our laboratory.

Applying the vapor-phase ammonolysis technique to the multipin system yields a potent tool for generating large numbers of peptide amides for bioscreening. The approach complements other applications of the multipin method, where either support-bound peptides,[4] C-terminal peptide acids,[32] or C-terminal diketopiperazine[8,20] peptides are generated for rapid and comprehensive screening studies.

Experimental Section

Carbon-13 NMR spectra were recorded at 50 MHz in DMSO-d_6 at 20 °C and are referenced to solvent at 39.5 ppm. Optical rotations were measured at 598 nm in a 10-cm path length cell. Melting points are uncorrected. Elemental analyses were performed by Chemical and Micro Analytical Services, North Essendon, Victoria, Australia. DMF was vacuum distilled from ninhydrin. Ether was distilled from sodium/benzophenone. EtOAc, TFA, and CH_2Cl_2 were distilled. AcOH, EtOH, MeOH, ^{i}PrOH, THF, and petroleum spirits (40–60 °C fraction, referred to here as petrol) were AR grade. Anisole, DCC, HOBt, DCHA, EDT, (*tert*-butoxycarbonyl)-1,6-diaminohexane, and zinc powder were from Fluka, Switzerland. DMAP was from Aldrich, Milwaukee, WI. Fmoc protected amino acids and PyBOP were from Novabiochem, Switzerland. Solutions were dried using anhydrous Na_2SO_4. NH_3/THF (3:7, v/v) was prepared by condensing NH_3(g) into THF cooled to −78 °C. NH_3/MeOH (3:7, v/v) and NH_3/^{i}PrOH (3:7, v/v) were similarly prepared. The solutions were stored in well-sealed bottles at −20 °C. Phenacyl glycolate (**13**), phenacyl 4-(hydroxymethyl)benzoate (**10**), (9-fluorenylmethoxycarbonyl)-L-prolylglycolic acid (DCHA salt) (**15b**), and *N*-(2,4-dinitrophenyl)-β-alanine (**20**) were prepared as previously reported.[20]

Phenacyl 4-[[[*N*-(9-Fluorenylmethoxycarbonyl)-*O*-*tert*-butyl-L-tyrosyl]oxy]methyl]benzoate (11q) (General Procedure). A stirring solution of Fmoc-L-Tyr(^{t}Bu)-OH (12.00 g, 26.1 mmol), **10** (7.06 g, 26.1 mmol), and DMAP (0.54 g, 4.44 mmol) in CH_2Cl_2 (200 mL) was cooled to 0 °C, and a solution of DCC (5.39 g, 26.1 mmol) in CH_2Cl_2 (30 mL) was added. The mixture was maintained at 0 °C for 2 h and then allowed to stir at ambient temperature for 18 h. The solution was filtered and evaporated, affording an oil, which was dissolved in EtOAc (300 mL). The solution was diluted with ether (150 mL) and washed sequentially with 10% citric acid, warm 4% NaOH (5×), and brine and dried. Evaporation gave a gum which crystallized upon trituration with ether, furnishing **11q** (18.48 g, 99%) as a white solid; mp 84–85 °C.

4-[[[*N*-(9-Fluorenylmethoxycarbonyl)-*O*-*tert*-butyl-L-tyrosyl]oxy]methyl]benzoic Acid (12q) (General Procedure). Zinc powder (12 g) was added to a stirring suspension of **11q** (18.00 g, 25.3 mmol) in EtOAc (60 mL), AcOH (140 mL), and water (60 mL). The mixture was heated to 60 °C for 16 h, filtered, and then evaporated. The residue was partitioned between EtOAc and hot water. The organic phase was washed sequentially with 10% citric acid and brine and dried. Evaporation of the solution afforded a gum which gave **12q** (13.68 g, 91%) as a white crystalline solid upon trituration with ether; mp 132–134 °C.

(9-Fluorenylmethoxycarbonyl)-L-isoleucylglycolic Acid, Dicyclohexylammonium Salt (15a) (General Procedure). A stirring solution of Fmoc-L-Ile-OH (15.71 g, 44.5 mmol), **13** (8.75 g, 45.0 mmol), and DMAP (1.123 g, 9.0 mmol) in CH_2Cl_2 (120 mL) was cooled to 0 °C and a solution of DCC (9.33 g, 45.0 mmol) in CH_2Cl_2 (30 mL) added. After 1 h the solution was allowed to warm to ambient temperature, and stirring was continued for 16 h. The reaction mixture was then filtered and evaporated. The resulting gum was dissolved in EtOAc (300 mL) and the solution washed sequentially with 10% citric acid/brine, 4% NaOH/brine (2×), 10% citric acid/brine, and brine and then dried. The gum obtained upon evaporation was triturated with ether to afford white crystals of **phenacyl (9-fluorenylmethoxycarbonyl)-L-isoleucylglycolate (14a)** (23.00 g, 98%). A solution of the product (22.40 g, 42.3 mmol) in EtOAc (60 mL) was mixed with AcOH (90 mL) and water (5 mL), zinc dust (10 g) was added, and the solution was stirred for 16 h. The

(31) Workers in our laboratory have found that T cells will tolerate 2% acetonitrile in aqueous buffer (pH 7.4) without adverse effect in *in vitro* assays. Mutch, D. A.; Underwood, J.; Geysen, H. M.; Rodda, S. J. Manuscript in preparation.

(32) Valerio, R. M.; Benstead, M.; Bray, A. M.; Campbell, R. A.; Maeji, N. J. *Anal. Biochem.* **1991**, *19*, 168–177.

solution was filtered and evaporated and the resulting gum partitioned between EtOAc (300 mL) and brine (300 mL). The organic phase was washed with brine and then dried. Evaporation gave a gum, which was dissolved in ether (150 mL), and a solution of DCHA (10 mL) in petrol (150 mL) was added. A gum precipitated and crystallized on standing. The white crystals were washed with ether/petrol (1:1) and dried to afford **15a** (25.9 g, 98%); mp 134–135 °C.

Preparation of Pins for Peptide Synthesis. Detachable polyethylene pin heads were radiation grafted with methacrylic acid/dimethylacrylamide.[33] The grafted pin heads were coupled with (*tert*-butoxycarbonyl)-1,6-diaminohexane and Boc deprotected with TFA. A limited amount of Fmoc-βAla-OH was coupled (100 or 200 nmol/pin), the unreacted amine capped by acetylation, and the Fmoc protection removed. Linker compounds **12** and **15** were coupled to the pins at this point. For peptide synthesis, the derivatized pin heads were fitted to polypropylene support shafts arranged in the 8 × 12 format used with the multipin system.

Peptide Synthesis. Peptides **16–19** were synthesized using N^{α}-Fmoc-protected amino acids. Amino acids requiring side-chain protection were as follows: Arg(Pmc), Asp(O^{t}Bu), Glu(O^{t}Bu), His(Boc), Lys(Boc), Ser(^{t}Bu), and Thr(^{t}Bu), Tyr(^{t}Bu). Coupling reactions were performed in polypropylene microtiter trays using 150 μL of activated amino acid solution per pin. Fmoc-Arg(Pmc)-OH and Fmoc-His(Boc)-OH were coupled using a solution containing protected amino acid (100 mM), PyBOP (100 mM), HOBt (100 mM), and NMM (150 mM) in DMF. All other coupling solutions contained protected amino acid (60 mM), DCC (60 mM), and HOBt (66 mM) in DMF. Couplings were performed at 25 °C for 4 h or 16 h. Following a wash with MeOH and air drying, the pins were Fmoc deprotected by treatment with 20% piperidine in DMF (50 mL per 96 pins) for 30 min. Deprotection was followed by a DMF wash, two MeOH washes, and air drying. This cycle was repeated until the target peptides were assembled. Peptides were side-chain deprotected with TFA/anisole/EDT (95:2.5:2.5, v/v/v) for 4 h at 20 °C. Following air drying, the pins were sonicated in 0.1% HCl in water/MeOH (1:1) (5 L) for 15 min using a 1000-W sonicator. The pins were then soaked in EtOH for 20 min and then air dried.

Peptide Cleavage and Elution. A bottle of NH_3/THF (3:7, v/v, 100 mL) was cooled to −78 °C, opened, and placed in a well-greased desiccator together with one to four multipin pin holders (each containing 96 pins). A partial vacuum was applied, the desiccator lid clamped, and the solution allowed to warm to ambient temperature. After 24 h, the multipin pin holders were removed from the desiccator and allowed to stand for 30 min. Elution method A: peptides were eluted by immersing the mounted pin heads into acetonitrile/water (40:60, v/v) (150 μL/pin) contained in the wells of a 96-well polypropylene microtiter tray for 3 h. Elution method B: peptides were eluted into acetonitrile/water (40:60, v/v) (400 μL/pin) in racked micro test tubes (Biorad) contained in an 8 × 12 rack immersed in a 1000-W sonication bath; the pins were sonicated for 30 min.

Model Cleavage Studies. Pins derivatized with **12a-s** or **15a-c** were Fmoc deprotected, washed, and dried as described above. The pins were treated with a solution of **20** (100 mM), PyBOP (100 mM), HOBt (100 mM), and NMM (150 mM) in DMF in the wells of a poly(propylene) microtiter tray for 16 h. Following washing, the pins were subjected to side-chain deprotection, sonication, and soaking in EtOH as described above. Cleavage was performed by storing the pins over NH_3/THF (3:7, v/v), NH_3/MeOH (3:7, v/v), or NH_3/^{i}PrOH (3:7, v/v) for 24 h. Elution method B was used. Following elution, the pins were washed and then subjected to a second 1-h cleavage with 2% NaOH in EtOH/water (1:1, 600 μL/pin) under the action of sonication. Absorbances of the elution solutions and the base-cleavage solutions were determined at 405 nm in polystyrene microtiter plates on an automated EIA plate reader (Whittaker M. A. Bioproducts, MA 310). The cleavage efficiency (X) was thus calculated:

$$X = \frac{100\text{Abs(1st elution)}}{\text{Abs(first elution)} + \text{Abs(second cleavage)}}$$

Racemization Studies. Two sets of pins derivatized with **12** were Fmoc deprotected, washed, dried, and coupled with Fmoc-L-Leu-OH and Fmoc-D-Leu-OH, respectively. Following Fmoc deprotection, washing, and drying, the pins were stored over NH_3/THF (3:7, v/v) for 24 h. The dipeptides were eluted using method B and subjected to HPLC analysis using the system described below. The 214-nm peak areas for the respective isomers were compared to determine the level of racemization.

Peptide Appraisal. Analytical HPLC was performed at 30 °C on a Waters Associates HPLC comprising two 510 pumps, a WISP-12B autosampler, and a Model 440 UV detector (254 nm) with an extended wavelength module (214 nm). Data and equipment was managed by Waters Maxima chromatography software. A 5-μm Merck Lichrosphere 100 RP-18 column was used. Gradient elution from A (0.1% TFA in water) to B (0.1% TFA in water/acetonitrile (40:60)) at 5–20 min. Amino acid analysis was performed as described previously.[8] Positive-ion FAB and CI-CH_4 mass spectra were recorded on a Finnigan Mat TSQ 46C mass spectrometer fitted with a FAB source and a CI source, respectively. Ion spray mass spectroscopy was performed on a Perkin-Elmer Sciex API-III spectrometer.

Acknowledgment. The authors wish to thank Megan Jay and David Stanton for their skilled technical assistance. Mass spectra were recorded by Dr. Paul Alewood and Alun Jones (University of Queensland) and Bruce Tattam (University of Sydney). NMR spectra were recorded by Dr. Ilias Kyratzis (Swinburne University of Technology).

Abbreviations: βAla, β-alanine; Axx, amino acid residue; DCHA, *N*,*N*′-dicyclohexylamine; Dnp, 2,4-dinitrophenyl; EDT, 1,2-ethanedithiol; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; MeCN, acetonitrile; MeOH, methanol; NMM, *N*-methylmorpholine; Pac, phenacyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; ^{i}PrOH, 2-propanol; pGlu, pyroglutamic acid; PyBOP, (benzotriazol-1-yloxy)tris-pyrrolidinophosphonium hexafluorophosphate; Trt, trityl. Nomenclature for α-amino acid residues is in accordance with IUPAC–IUB, Nomenclature and Symbolism for Amino Acids and Peptides. *Eur. J. Biochem.* **1984**, *138*, 9–37.

Supplementary Material Available: ^{13}C NMR data for compounds **12a-s**, and **15a-c** (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(33) Maeji, N. J.; Valerio, R. M.; Bray, A. M.; Campbell, R. A.; Geysen, H. M. *Reactive Polymers*, in press.