was removed and rotary evaporated and the residue Kugelrohr distilled to give 120 mg (4%), bp 115-120 °C. ^{1}H NMR: δ 1.31 (d, 3 H), 2.53 (m, 1 H), 2.64 (m, 1 H), 3.17 (m, 2 H).

1-Methyl-3-methylenecyclobutane (6). Dimethyl sulfoxide (distilled from CaH_2) was stirred under Ar for 30 min. Methyltriphenylphosphonium iodide (1.45 g, 3.6 mmol) was added and the reaction stirred for 2 h. 3-Methylcyclobutanone (120 mg, 3.6 mmol) was dissolved in 2 mL of DMSO and added to the reaction via syringe. A cannula was connected from the reaction flask to a cold trap cooled in dry ice–acetone. A small amount of liquid was collected, and NMR and GC–MS were taken. ^{1}H NMR:[13] δ 1.13 (d, 3 H), 2.24 (d, 2 H), 2.36 (m, 1 H), 2.80 (m, 2 H), 4.71 (d, 2 H). GC–MS showed M^+ at m/e 82.

Methylbicyclo[1.1.1]pentane (8).[6a] To crude 7 (5.1 mmol, 1.1 g) in a round-bottomed flask that had been flushed with N_2, cooled to −15 °C, and protected from light with Al foil was added thiophenol (10 mL, 0.1 mol) via syringe. The foil was removed and the stirred solution irradiated with a 300-W tungsten filament lamp for 30 min. A cannula was connected from the reaction flask to a cold trap cooled to −78 °C, and the reaction mixture was warmed to 70 °C. A small amount of clear distillate was collected (200 mg, 50%). ^{1}H NMR:[4] δ 1.1 (s, 3 H), 1.66 (s, 6 H), 2.48 (1 H, s). GC–MS showed M^+ peak at m/e 82.

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Registry No. 3, 136379-21-0; 6, 15189-18-1; 7, 136379-22-1; 8, 10555-48-3; 1-bicyclo[1.1.1]pentylacetic acid, 131515-31-6; *N*-hydroxy-2-(1*H*)-pyridinethione, 1121-30-8; 2-(2-methyl-3-chloropropyl)-1,3-dithiane, 53198-70-2; dithiane, 51330-42-8; 1-bromo-3-chloro-2-methylpropane, 6974-77-2; 3-methylcyclobutanone trimethylene thioketal, 136379-23-2; 3-methylcyclobutanone, 1192-08-1; methyltriphenylphosphonium iodide, 2065-66-9.

Direct Cleavage of Peptides from a Solid Support into Aqueous Buffer. Application in Simultaneous Multiple Peptide Synthesis[1]

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A method of simultaneous multiple peptide synthesis which integrates synthesis, side-chain deprotection, cleavage, and purification so as to afford peptide solutions suitable for immediate biological testing is described. The approach utilizes a novel diketopiperazine-forming cleavable linker **1**. Upon side-chain deprotection, **1** gives **2**, which is stable to a protocol designed to remove contaminants from the support-bound peptide prior to cleavage. Peptide cleavage is then effected by treating **2** with a neutral or near neutral buffer to give peptide **4**, which carries a C-terminal diketopiperazine moiety, in good yield. In this study the glycolamido and 4-(oxymethyl)benzamido esters of **1** have been appraised. The approach is demonstrated in model studies on **7** and **8** and in the preparation and characterization of peptides **17–21**. The general approach allows 10–100-nmol quantities of many hundreds of peptides to be concurrently prepared in a relatively short period of time when used in conjunction with the multipin method of multiple peptide synthesis.

Introduction

Growth in the demand for synthetic peptides has been partly addressed by a range of techniques facilitating rapid peptide synthesis through parallel handiing. Simultaneous multiple peptide synthesis has been performed on resin,[2-5] cellulose[6,7] and grafted polyethylene[8-10] or polypropylene[11] supports. Despite the speed at which peptides can be assembled by a parallel synthesis strategy, the need for individual handling at the side-chain deprotection, cleavage, and purification steps limits the number of peptides that can be conveniently prepared. Several methods of overcoming the postsynthesis bottleneck have been proposed. For example, closely related peptides can be synthesized on the same support and subsequently separated by HPLC.[4] Purification and characterization must be straightforward, however, if this method is to succeed. The use of specialized apparatus designed for multiple peptide cleavage simplifies cleavage and side-chain deprotection[12]

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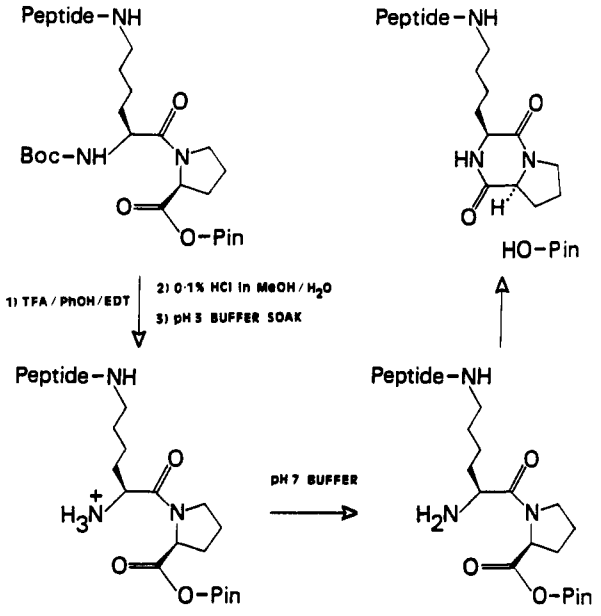
Scheme I. Peptide Cleavage via Diketopiperazine Formation



but does not obviate the need for individual peptide purification. The multipin approach of Geysen[8,9] bypassed the need for individual handling by synthesizing peptides on plastic pins arranged to complement the 96-well microtitre plate used in enzyme-linked immunosorbent assay (ELISA). By using the same support for both synthesis and testing, the screening of large numbers of peptides by ELISA was greatly simplified. Peptides bound to a solid support, however, have limited application. Until recently,[1,13] no method of simultaneous synthesis integrated peptide assembly, side-chain deprotection, cleavage, and purification so as to allow immediate biological testing.

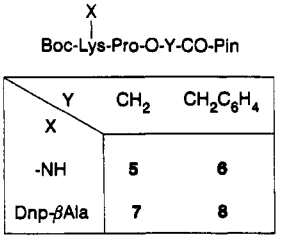
An integrated approach requires that the following strategy be adopted. Side-chain deprotection and peptide cleavage must be distinct processes, separated by a rigorous washing protocol designed to remove all organic contaminants, which may prove toxic in subsequent assays. Peptide purity is then dependent solely on the efficiency of peptide synthesis and side-chain deprotection. The second requirement is that side-chain-deprotected peptides can be cleaved directly into a medium compatible with their final use. Generally, this would be aqueous. Postcleavage handling is then minimal and a fully integrated method for the simultaneous handling of large numbers of discrete peptides becomes viable.

A linker which meets the dual requirement of stability under the conditions of peptide synthesis and side-chain deprotection and lability under biologically compatible conditions has been designed. As shown in Scheme I, a diketopiperazine-forming linker (1) is interposed between the solid support and the target peptide. Side-chain deprotection primes the linker for cleavage with the formation of ammonium salt 2. Provided the assembly remains in the protonated form, it is stable to washing steps in organic solvents, aqueous/organic solvents, and low pH buffers. With the equilibrium generation of 3 in neutral aqueous buffer, cyclization, and hence cleavage to give 4, proceeds with good efficiency. The resulting peptides carry a diketopiperazine moiety at the C-terminal.

In this study, the strategy outlined above has been explored in conjunction with the multipin method of peptide synthesis. Studies on model system 1, where "Peptide" = (2,4-dinitrophenyl)-β-alanine (Dnp-βAla), show that 1 is stable to the conditions of peptide synthesis, 2 is stable to the precleavage washing protocol, and cleavage proceeds rapidly and efficiently in pH 7 phosphate buffer. The utility of the approach has also been demonstrated with the synthesis and characterization of a selection of test peptides. This approach has allowed us to concurrently prepare thousands of discrete solution-phase peptides in a matter of weeks.[1,13] As the peptides are produced in the 10–100-nmol range, they may be characterized by conventional techniques. These quantities are adequate for many biological, immunological, and pharmacological applications. The peptides are suitable for applications where native C-termini are not required.

Results

Design and Preparation of Diketopiperazine-Forming Handle 1. A diketopiperazine-forming cleavable linker such as 1 has two requirements: a C-terminal *N*-alkylamino acid and an amino acid with a side-chain functional group upon which peptide synthesis can be performed. Rather than proline, another *N*-alkylamino acid such as sarcosine could have been used. The rate of diketopiperazine formation is, however, dependent on the *N*-alkylamino acid.[14] Lysine was chosen as the second residue so that peptide synthesis could be continued on the side chain. The protecting groups in 1 were arranged for use with N^{α}-Fmoc-protected amino acids with acid-labile side-chain protection. The stability/lability properties of the linker can also be expected to be sensitive to the type of ester linkage to the solid support.[15,16] In early work,[13] 1 was assembled on the side chain of serine. In this study linkers 5 and 6 based respectively on the glycolamido[14] and 4-(oxymethyl)benzamido[15] esters of lysylproline have been investigated.



Rather than assemble the linker in piecemeal fashion as was done in earlier work,[13] the ester bond was formed prior to coupling to the pin surface, hence bypassing the need to perform a moisture-sensitive dicyclohexylcarbodiimide (DCC)/4-(dimethylamino)pyridine (DMAP) mediated coupling in an open container. Linker 5 was assembled onto the pins in two coupling steps using preformed ester 11 and Boc-Lys(Fmoc)-OH. The dicyclohexylamine (DCHA) salt of compound 11 was prepared in 91% overall yield as shown in Scheme II. The linker assembly was simplified further by incorporating 6 onto the pin in one step using dipeptide ester 16. An overall yield of 64% was achieved in the five-step synthesis of 16, presented in Scheme III.

Stability and Cleavage Studies. As thousands of peptides are handled simultaneously, the method of syn-

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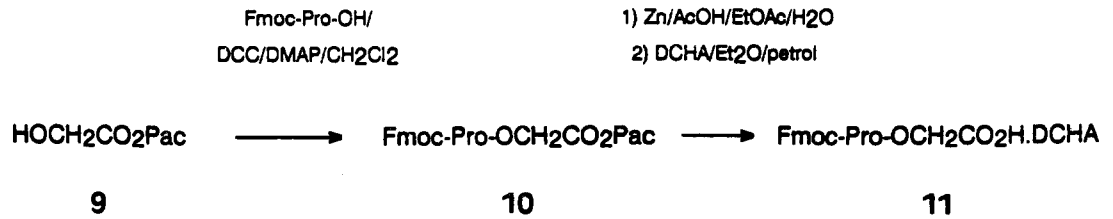
Scheme II. Synthesis of Compound 11 (Pac = phenacyl ($CH_2COC_6H_5$))



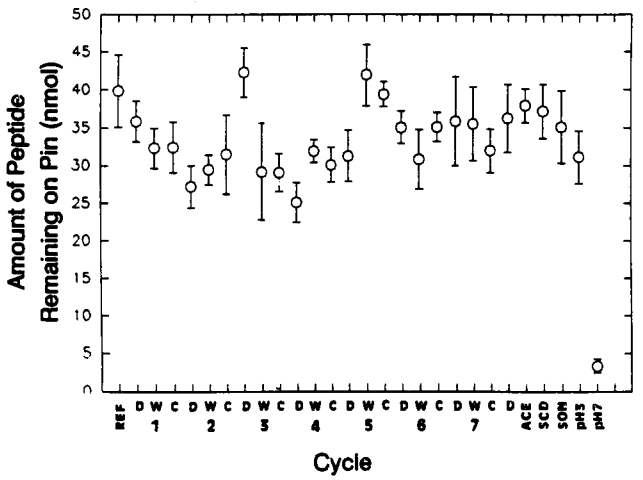


Figure 1. Stability of Boc-Lys(Dnp-βAla)-Pro-OCH_2CO-pin (7) during peptide synthesis. After each discrete step six pins were removed and cleaved with 0.25 M NaOH, and the amount of material cleaved from the pin was determined. D, deprotection with 20% piperidine in DMF; W, wash with MeOH; C, dummy coupling with Fmoc-Gly-OH/DCC/HOBt/DMF; A, acetylation; SCD, side-chain deprotection with TFA/PhOH/$HS(CH_2)_2SH$ (95:2.5:2.5, v/w/v); SON; sonication in 0.1% HCl in MeOH/H_2O (1:1, v/v); pH3, precleavage soak in pH 3 buffer; pH7, cleavage with pH 7 buffer.

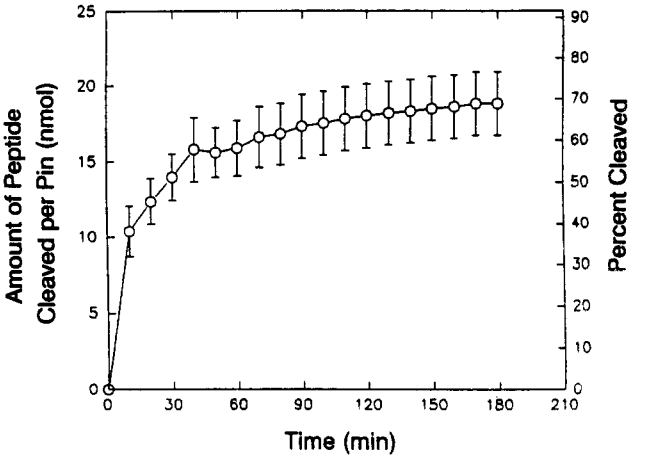


Figure 2. Cleavage of H-Lys(Dnp-βAla)-Pro-OCH_2CO-pin with 0.1 M pH 7 phosphate buffer. Each data point is an average of 30 pins.

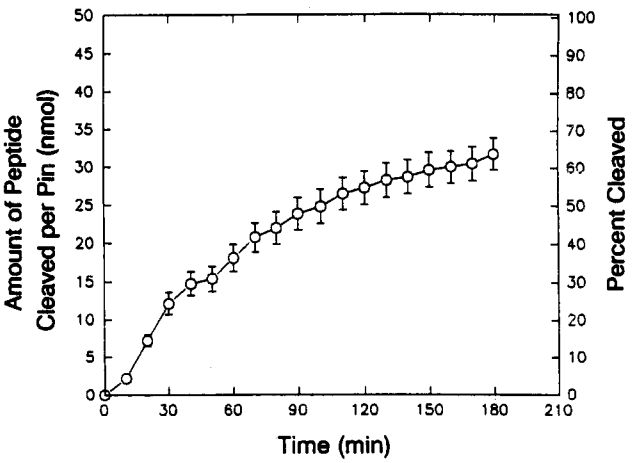


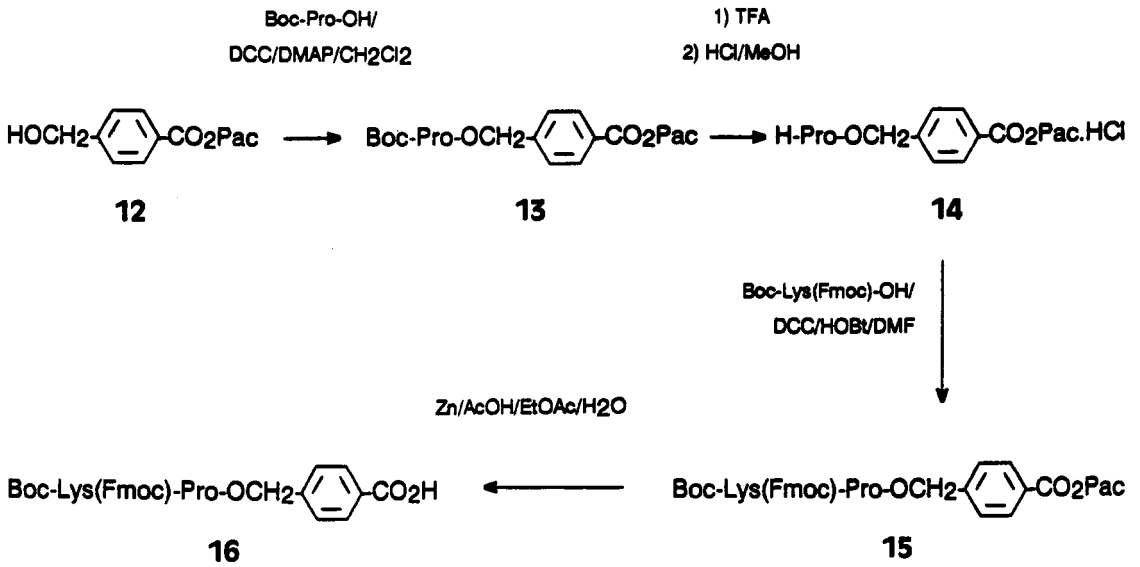
Figure 3. Cleavage of H-Lys(Dnp-βAla)-Pro-$OCH_2C_6H_4CO$-pin with 0.1 M phosphate buffer (pH 7). Each data point is an average of 28 pins.

thesis used in multipin synthesis deviates, by necessity, from conventional solid-phase peptide synthesis. Coupling reactions are performed for 16 h under nonanhydrous conditions. MeOH is used in postcoupling and postdeprotection washing steps and washes are performed in open containers. Although these conditions appear not to compromise peptide quality, the stability of the ester link to these conditions was unknown. Hence the stability of the glycolamido and 4-(oxymethyl)benzamido ester groups were reassessed using the model systems **7** and **8** to demonstrate compatibility with the Fmoc synthesis strategy as applied to pins. A stability study performed on **7** is summarized in Figure 1. One hundred and fifty-six derivatized pins were subjected to a mock heptapeptide synthesis in which six pins were removed for analysis followed each discrete step. Analysis comprised trifluoroacetic acid (TFA) treatment of the pins followed by cleavage into the wells of a microtitre plate with 0.25 M NaOH (3 h) and subsequent spectrophotometric determination of the cleavage solutions. Less than 5% of the model system was lost from the pin surface over seven deprotection/wash/coupling cycles. In practice, occasions have been found where inadvertant cleavage of the glycolamido linker **5** has occurred during peptide synthesis (unpublished results). In contrast, the benzamido linker **6** has superior stability. Sporadic loss of this system from the pin through synthesis has not been encountered; consequently **6** has been adopted for most of our routine work.

A two-step post-side-chain deprotection protocol was developed earlier for use with a diketopiperazine-forming cleavable linker assembled on the side chain of serine.[13] The protocol was designed to afford nontoxic peptide solutions free of organic contaminants upon cleavage. Sonication of the pins in 0.1% HCl in MeOH–water effectively removes scavengers and byproducts arising from side-chain deprotection. Counterion exchange of trifluoroacetate for chloride also occurs in this step. MeOH and any remaining organic contaminants are then removed in a subsequent low pH aqueous buffer soak. Previously,[13] a 16-h soak in 0.1 M pH 5 phosphate buffer was advocated. Alternatives to this procedure have been examined in order to reduce the risk of inadvertant peptide cleavage. A 1-h pH 5 soak has been employed but is not favored in the preparation of peptides for use with sensitive-cell mediated assays. The preferred method and the primary one used in this study is a 5-h soak in pH 3 citrate–phosphate buffer.

The rate and efficiency of the buffer-mediated cyclization–cleavage step was also investigated. Figure 2 shows the results of a time trial cleavage study performed on 30 pins derivatized with model system **7**. Following treatment with TFA, sonication, and a 1-h precleavage soak in pH

Scheme III. Synthesis of Compound 16



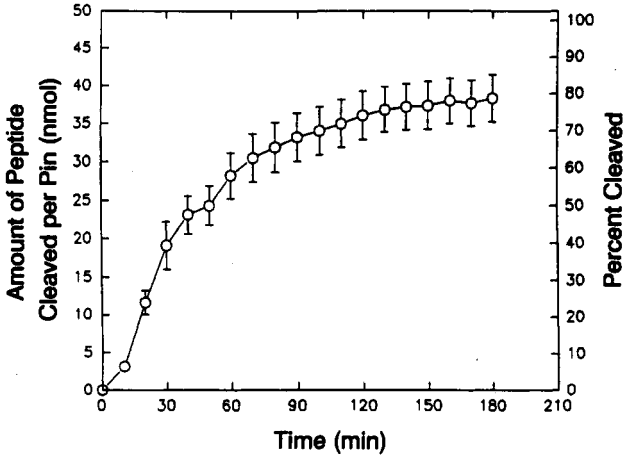


Figure 4. Cleavage of H-Lys(Dnp-βAla)-Pro-$OCH_2C_6H_4CO$-pin with 0.1 M $NaHCO_3$ (pH 8.3). Each data point is an average of 28 pins.

5 buffer, each pin was immersed in 150 μL of a 0.1 M pH 7 phosphate buffer solution within a 96-well microtitre plate. Absorbances of the cleavage solutions were read every 10 min over a 3-h period. Within 3 h, 70% cleavage was achieved, yielding 150 μL of a 0.17 mM solution of cleaved peptide per pin. Two similar studies, summarized in Figures 3 and 4, were performed on model system 8. In these studies the alternative 5-h, pH 3 precleavage soak was used. Twenty-eight pins were treated with 0.1 M pH 7 phosphate buffer for 3 h, after which time 64% of the available peptide was cleaved. Twenty-eight pins were cleaved with 0.1 M $NaHCO_3$ to demonstrate the use of other near neutral cleavage media. In this case 79% cleavage was effected after 3 h. Cleavage with 0.25 M NaOH for 3 h removed all of the color from the pin surface and was taken to be 100% cleavage. This was confirmed by amino acid analysis on the cleaved pins. When water was substituted for the cleavage buffer, less than 2% cleavage was observed at 3 h.

Application in Peptide Synthesis. Five decapeptide sequences, shown in Table I, were selected for critical appraisal of the cleavage method used in conjunction with the multipin method of peptide synthesis. Peptides were prepared on pins derivatized with the linker system **5**. A β-alanine (βAla) spacer was included between the linker and target peptide. The peptide set included two common test sequences, acyl carrier protein (ACP) 65–74[18] **19** and

Table I. Peptides Used in Method Appraisal

	sequence	amount cleaved[a] (nmol)	positive ion FAB MS data[b] (*m/z*)
17	Ac-ASQGGLEDPA-βAla-cyclo(KP)	61	1309, $[M - H + 2Na]^+$; 1287, $[M + Na]^+$
18	Ac-PGPSDTPILP-βAla-cyclo(KP)	66	1336, $[M + Na]^+$
19	Ac-VQAAIDYING-βAla-cyclo(KP)	40	1428, $[M - H + 2Na]^+$; 1406, $[M + Na]^+$
20	Ac-WEIPEPYVWD-βAla-cyclo(KP)	40	
21	Ac-SYSMEHFRWG-βAla-cyclo(KP)	18	

[a] Analysis performed on the cleavage product from a single pin. [b] Not obtained in **20** and **21**.

adrenocorticotrophic hormone (ACTH) 1–10[19] **21**, which are widely used in the appraisal of automated peptide synthesizers. The peptides were synthesized using an Fmoc synthesis protocol, where couplings were effected using DCC/1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Following N-terminal capping by acetylation, the peptides were side-chain deprotected with TFA/phenol/ethanedithiol (EDT). Following sonication and a 5-h precleavage soak in pH 3 buffer, the peptides were cleaved from the pins into 150 μL of 0.1 M pH 7 phosphate buffer in the wells of a microtitre plate.

Peptides were examined by reverse phase HPLC, amino acid analysis, and in selected cases positive ion FAB mass spectrometry. Figure 5 presents chromatograms of four of the test peptides recorded at 214 nm. None of these peptides were subjected to prior purification. In each case a single major peak was observed. In all cases a minor peak due to phenol, one of the scavengers used in side-chain deprotection, was observed at t_R 15.77 min. The methionine-containing peptide **21** was obtained in both the oxidized and reduced forms. Aerial oxidation of the solution in the presence of base afforded a product of t_R 18.13 min. Amino acid ratios were determined for all five cleaved peptides and are presented in Table II. Analyses were performed on solutions of high salt content and low peptide content, nonideal conditions for peptide hydrolysis

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Table II. Amino Acid Analysis of Peptides Cleaved into 0.1 M Phosphate Buffer (pH 7.0)

	ratios[a]				
amino acid	17	18	19	20	21
A	2.0 (2)		1.8 (2)		
D	0.7 (1)	0.7 (1)	1.6 (2)	0.8 (1)	
E	2.1 (2)		0.7 (1)	1.8 (2)	0.9 (1)
F					1.0 (1)
G	1.4 (2)	0.6 (1)	0.9 (1)		0.8 (1)
H					0.9 (1)
I		1.0 (1)	1.9 (2)	1.0 (1)	
K[b]	0.7 (1)	0.7 (1)	1.1 (1)	1.2 (1)	1.1 (1)
L	1.2 (1)	1.0 (1)			
M					1.1 (1)
P[b]	3.0 (2)	6.5 (5)	1.6 (1)	3.7 93)	1.8 (1)
R					1.0 (1)
S	0.6 (1)	0.6 (1)			1.1 (2)
T		0.8 (1)			
V			0.9 (1)	0.8 (1)	
W				1.8 (2)	1.0 (1)
Y			1.0 (1)	0.8 (1)	1.1 (1)
βAla[b]	1.3 (1)	1.1 (1)	1.5 (1)	1.1 (1)	1.4 (1)

[a] Values in parentheses are expected values. [b] Residues in linker.

and phenyl isothiocyanate (PITC) derivatization.[20,21] Nevertheless, the analysis results give ratios consistent with the target sequences. The quantity of peptide cleaved from the pin support is listed in Table I. Typically, cleavage solutions contain between 20 and 60 nmol of peptide. Variation in the individual loading of the pins reflects the discrete nature of this solid support. The identity of three of the peptides was further confirmed by positive ion FAB mass spectrometry; the data are included in Table I. Due to the high sodium concentration of the cleavage solution, $[M + Na]^+$ rather than $[M + H]^+$ signals were observed.

Discussion

In general, peptides prepared by conventional solid-phase synthesis strategies are side-chain deprotected and cleaved from the solid support in a single step. This necessitates individual purification of crude peptide, usually by preparative HPLC. Once pure, the peptide is further handled in preparation for biological assay. This type of approach is not practical when handling small quantities of thousands or even hundreds of peptides. The challenge is to devise strategies where large numbers of peptides can be synthesized, side-chain deprotected, purified, cleaved, and presented for biological testing in a simultaneous fashion.

The propensity of resin-bound dipeptide esters with a C-terminal *N*-alkylamino acid residue to undergo intramolecular aminolysis was recognized in the early 1970s.[22-24] Diketopiperazine formation is a side reaction where quantitative loss of peptide from resin can occur under very mild conditions. However, provided the deprotected dipeptide remains protonated as in **2**, it is relatively stable. As the rate of cyclization is dependent on the *N*-alkylamino acid[14] and on the type of peptide–ester linkage to the solid support,[15,16] there is scope to tune the reaction to a desired set of cleavage conditions. Though regarded as a problem reaction, it satisifies the criteria required for a buffer-labile cleavage reaction.

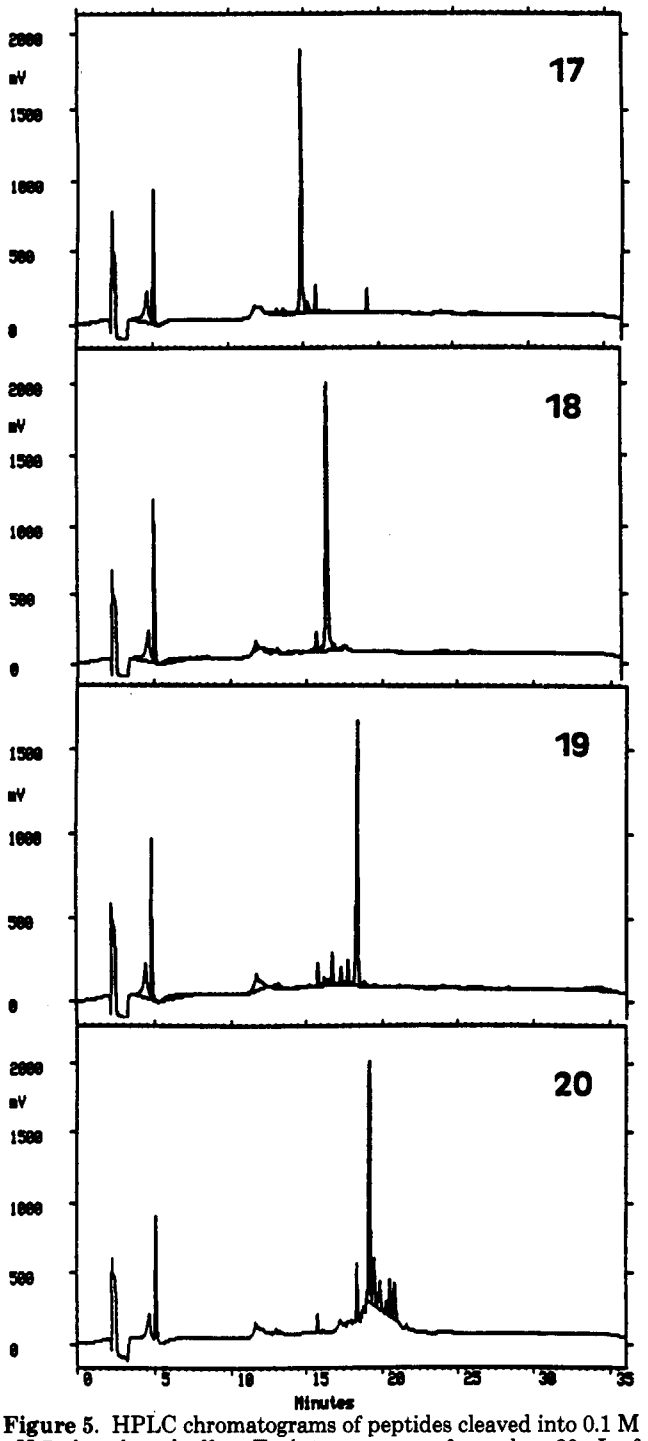

Figure 5. HPLC chromatograms of peptides cleaved into 0.1 M pH 7 phosphate buffer. Each trace was performed on 80 μL of the cleaved peptide solution. Detection at 214 nm. Solvent A, H_2O (0.1% TFA); solvent B, 60% CH_3CN (0.1% TFA). Linear gradient A to B from 5 min to 20 min. Column: Merck LiChrosphere 100 RP-18 5 μm.

Using the lysylproline assembly presented in Scheme I, cyclization, and hence cleavage, could be triggered by a pH 7 buffer solution. In practice, the cleavage properties of **5** and **6** were found to be similar. In preliminary work, where the cleavable linker was assembled on the side chain of serine, cleavage was shown to take place in phosphate buffer solutions of pH 6 to pH 8 over a range of ionic strengths.[13] Other near neutral buffer solutions have also proved useful. As expected,[23] cyclization was found not to occur to an appreciable extent during post-side-chain deprotection steps, where the assembly **2** was maintained in a protonated form. This is the first described integrated

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approach allowing the simultaneous handling of peptides through synthesis, side-chain deprotection, cleavage, and presentation for assay. When used in conjunction with the multipin method of peptide synthesis, the handling of thousands of discrete peptides becomes practical.

We routinely examine cleaved peptide solutions by HPLC and amino acid analysis, and in many cases results comparable to those obtained for analogous peptides produced by conventional resin-based synthesis have been achieved. Furthermore the potency of peptides produced by this method has been demonstrated in T-cell proliferation assays.[13,25] It is useful, however, to consider potential limitations of the method. In common with other approaches where extensive individual purification is not practiced, difficult sequences can be expected to be of lower purity than easy sequences. Whether or not this is acceptable depends on the nature of the end use of the peptide. Prolonged standing of peptides in neutral solutions during cleavage results in the partial oxidation of Met and Cys residues.[26] This is common, however, to any test situation where peptides are handled at or near neutral pH. Although diketopiperazines are prone to epimerization,[27] this has only been observed with cleavage into aqueous NaOH;[13] it has not been encountered with neutral buffers. One strength of the method is also a potential weakness: very lipophilic peptides may not be efficiently extracted from the solid support by a wholly aqueous cleavage medium. If organic additives such as ethanol or acetonitrile are compatible with the assay system, these can be added to assist peptide dissolution. In the work presented here, this has not been necessary.

There are instances where C-terminal carboxylate or carboxamide groups are mandatory, such as in the study of small hormones. Nevertheless, native C-terminal groups are not always required. For example, the method has been applied successfully in the area of T-cell determinant analysis,[13,25] in the production of peptide–protein conjugates,[28] and in methodological studies. In these applications the presence of an unnatural C-terminal group has not been found to be detrimental. The challenge now is to devise similar methods where peptides with native C-termini can be prepared in a conceptually similar manner. Procedures yielding peptides with C-terminal carboxylate and carboxamido groups are currently being evaluated in our laboratory and will be reported elsewhere.[29,30]

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. Unless otherwise stated, optical rotations were recorded in DMF at c 1.0. Melting points are uncorrected. Elemental analyses were performed by Chemical and Micro Analytical Services, North Essendon, Victoria, Australia. TLC was performed on 5 × 20 cm glass plates coated with Kieselgel 60 F254 (Merck). Plates were developed in solvent A ($CHCl_3$/MeOH/AcOH (45:4:1)) or solvent B (CH_2Cl_2/MeOH/AcOH (90:4:1)) and visualized with 1% ninhydrin in MeOH/AcOH (99:1) or by viewing under 254-nm UV light as appropriate. DMF was vacuum distilled from ninhydrin. Ether was distilled from sodium/benzophenone. EtOAc, TFA, and CH_2Cl_2 were distilled. Dioxan, AcOH, MeOH, and petroleum spirits (40–60 °C fraction, hereon refered to as petrol) were of AR grade. Et_3N and N-methylmorpholine (NMM) were distilled from calcium hydride. DCC, HOBt, DCHA, phenacyl bromide (Pac-Br), phenol, and EDT were from Fluka, Switzerland. DMAP and 2,4-dinitrofluorobenzene (FDNB) were purchased from the Aldrich Chemical Company, Milwaukee, WI. 4-(Hydroxymethyl)benzoic acid (HMB), Boc-L-Lys(Fmoc)-OH, Boc-L-Pro-OH, and Fmoc-protected amino acids were from Novabiochem, Switzerland. Glycolic acid (Glyc) was from Sigma, St. Louis, MO. Solutions were dried using anhydrous Na_2SO_4.

Phenacyl Glycolate (9). Et_3N (14.0 mL, 100 mmol) and Pac-Br (20.0 g, 100 mmol) were added to a stirring suspension of glycolic acid (7.6 g, 100 mmol) in EtOAc (300 mL). After 20 h, the solution was diluted with warm EtOAc (300 mL) and extracted with warm water (300 mL). The organic phase was washed sequentially with 10% citric acid, 7% $NaHCO_3$, and brine and dried. The solid obtained upon evaporation was triturated with ether, affording **9** (13.51 g, 70%) as a white crystalline solid: mp 105–108 °C; R_f(A) 0.51; ^{13}C NMR δ 193.2, Pac CO; 172.6, Glyc CO; 134.3, Pac C4; 134.1, Pac C1; 129.2, Pac C2; 128.1, Pac C3; 66.5, Pac CH_2; 59.5, Glyc CH_2.

Anal. Calcd for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 61.45; H, 4.93.

(9-Fluorenylmethoxycarbonyl)-L-prolylglycolic Acid, Dicyclohexylammonium Salt (11). A stirring solution of Fmoc-L-Pro-OH (15.00 g, 44.5 mmol), **9** (8.75 g, 45.0 mmol), and DMAP (1.123 g, 9.0 mmol) in CH_2Cl_2 (150 mL) was cooled to 0 °C and DCC (9.33 g, 45.0 mmol) added. After 4 h, the solution was allowed to warm to ambient temperature and stirring was continued for 16 h. The solution was filtered and evaporated. The resulting gum was dissolved in EtOAc (300 mL) and the solution washed sequentially with 10% citric acid, 4% NaOH, 10% citric acid, and brine and then dried. Evaporation afforded **phenacyl (9-fluorenylmethoxycarbonyl)-L-prolylglycolate (10)** (23.02 g, 100%) as a pale yellow oil; R_f(A) 0.81. A solution of the product in EtOAc (50 mL) was mixed with AcOH (200 mL) and water (50 mL). Zinc dust (10 g) was added and the solution stirred for 16 h. The solution was filtered and evaporated and the resulting solid partitioned between EtOAc (300 mL) and hot water (300 mL). The organic phase was washed with brine, 10% citric acid, and brine and then dried. Evaporation afforded a gum, which was dissolved in ether (75 mL), and a solution of DCHA (8.16 g, 45.0 mmol) in petrol (50 mL) was added. A white gum precipitated and rapidly crystallized. The crystals were washed with ether (3 × 50 mL) and dried, affording **11** (23.25 g, 91%) as white crystals; mp 145–146 °C; R_f(A) 0.61; $[\alpha]^{21}_D$ −48.3° (c 1, MeOH); ^{13}C NMR δ 172.3, 172.0, Pro CO; 169.6, Glyc CO; 154.3, 154.1, Fmoc CO; 144.2, Fmoc C; 141.1, Fmoc C; 127.9, Fmoc C; 127.4, Fmoc C; 125.4, Fmoc C; 120.4, Fmoc C; 66.9, 66.7, Fmoc CH_2; 63.5, 63.4, Glyc CH_2; 59.0, 58.4, Pro Cα; 51.8, DCHA C1; 46.8, Pro Cδ; 46.7, Fmoc CH; 46.2, Pro Cδ; 30.1, Pro Cβ; 29.1, DCHA C2; 24.9, DCHA C4; 24.1, DCHA C3; 23.6, 22.6, Pro Cγ.

Anal. Calcd for $C_{34}H_{44}N_2O_6$: C, 70.80; H, 7.69; N, 4.86. Found: C, 70.77; H, 7.57; N, 4.79.

Phenacyl 4-(Hydroxymethyl)benzoate (12). A solution of Pac-Br (32.9 g, 165 mmol) and Et_3N (23.0 mL, 165 mmol) in EtOAc (100 mL) was added to a suspension of HMB (25.0 g, 164 mmol) in EtOAc (500 mL). After being stirred for 64 h, the reaction mixture was partitioned between warm water (300 mL) and EtOAc (300 mL). The organic phase was sequentially washed with 10% citric acid, 7% $NaHCO_3$, and brine and dried. The solid obtained upon evaporation was suspended in ether (150 mL) for 30 min and then collected by filtration. The solid was washed with ether and dried to yield **12** (35.95 g, 81%) as a white crystalline solid: mp 114–115 °C; R_f(A) 0.88, R_f(B) 0.69; ^{13}C NMR δ 194.1, Pac CO; 166.3, HMB CO; 149.6, HMB C1; 134.8, Pac C4; 134.7, Pac C1; 130.0, HMB C2; 129.7, Pac C2; 128.5, Pac C3; 128.2, HMB C4; 127.1, HMB C3; 67.3, Pac CH_2; 62.6, HMB CH_2.

Anal. Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found C, 71.02; H, 5.21.

Phenacyl 4-[[[(*tert*-Butoxycarbonyl)-L-prolyl]oxy]methyl]benzoate (13). A stirring solution of Boc-L-Pro-OH

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(19.38 g, 90 mmol), **12** (24.39 g, 90 mmol), and DMAP (1.86 g, 15.3 mmol) in CH_2Cl_2 (500 mL) was cooled to 0 °C and a solution of DCC (18.57 g, 90 mmol) in CH_2Cl_2 (100 mL) was added over 5 min. The mixture was maintained at 0 °C for 2 h and then allowed to stir for 18 h at ambient temperature. The reaction mixture was filtered and evaporated to yield an oil. The oil was dissolved in EtOAc (600 mL) and washed sequentially with 10% citric acid/brine (2:1), 4% NaOH/brine (2:1), and brine and dried. The oil obtained upon evaporation was dissolved in ether (100 mL) and the resulting solution filtered and evaporated to afford **13** (41.84 g, 100%) as a clear gum; $[\alpha]^{21}_D$ 23.9 °C; R_f(B) 0.95; ^{13}C NMR δ 194.0, Pac CO; 173.7, 173.4, Pro CO; 166.0, HMB CO; 153.8, Boc CO; 142.8, 142.6, HMB C1; 134.8, Pac C1; 134.7, Pac C4; 130.3, HMB C2; 129.7, Pac C2: 128.8, HMB C4; 128.6, Pac C3; 128.3, HMB C3; 79.4, 79.2, Boc Ct; 67.5, Pac CH_2; 65.5, 65.3, HMB CH_2; 58.8, Pro Cα; 46.4, 46.2, Pro Cδ; 30.2, 29.3, Pro Cβ; 28.0, 27.7 Boc CH_3; 23.9, 23.0, Pro Cγ.

Anal. Calcd for $C_{26}H_{29}NO_7$: C, 66.79; H, 6.25; N, 3.00. Found: C, 66.62; H, 6.37; N, 2.93.

Phenacyl 4-[(L-Prolyloxy)methyl]benzoate Hydrochloride (14). A solution of **13** (84.10 g, 180 mmol) in TFA (250 mL) was stirred for 3 h and then evaporated to afford a yellow oil. The oil was mixed with a solution of hydrogen chloride (13.13 g, 360 mmol) in MeOH (300 mL) and a white crystalline solid rapidly formed. After 10 min, the product was collected by filtration and washed sequentially with MeOH (2 × 100 mL) and ether (3 × 50 mL). A second crop obtained by evaporation of the filtrate was triturated with ether and collected by filtration. The product was air dried and then dried in vacuo over P_2O_5 to afford **14** (72.49 g, 99.7%) as a white crystalline solid: mp 170–172 °C; $[\alpha]^{21}_D$ −24.2 °C; R_f(B) 0.33; ^{13}C NMR δ 194.3, Pac CO; 170.0, Pro CO; 166.4, HMB CO; 142.2, HMB C1; 135.2, Pac C4; 135.0, Pac C1; 130.6, HMB C2; 130.1, Pac C2; 129.2, Pac C3; 128.9, HMB C3; 67.8, Pac CH_2; 67.0, HMB CH_2; 59.0, Pro Cα; 45.6, Pro Cδ; 27.9, Pro Cβ; 23.2, Pro Cγ.

Anal. Calcd for $C_{21}H_{22}ClNO_5$: C, 62.45; H, 5.49; N, 3.47. Found: C, 62.30; H, 5.53; N, 3.41.

Phenacyl 4-[[[N^{α}-(*tert*-Butoxycarbonyl)-N^{ϵ}-(9-fluorenylmethoxycarbonyl)-L-lysyl-L-prolyl]oxy]methyl]benzoate (15). Finely powdered **14** (35.70 g, 88.4 mmol) was added to a stirring solution of Boc-L-Lys(Fmoc)-OH (39.26 g, 84.0 mmol), DCC (18.24 g, 88.4 mmol), HOBt (13.52 g, 88.4 mmol), and NMM (8.942 g, 88.4 mmol) in DMF (500 mL). The solution was allowed to stir for 16 h. The reaction mixture was filtered, the precipitate was washed with EtOAc (80 mL), and the combined filtrate was evaporated. The crude product was dissolved in EtOAc (600 mL) and washed with warm 10% citric acid. The organic phase was then divided into two equal portions and each treated as follows. The solution (300 mL) was diluted with warm ether (900 mL) and then extracted with a 40 °C solution of 4% NaOH (6 × 200 mL). The organic phase was then washed with a 40 °C solution of brine. The combined, dried organic phase was left to stand for 16 h. The resulting crystalline solid was collected by filtration, washed with ether (100 mL), and then dried in vacuo over P_2O_5. A second crop obtained from the filtrate was also washed with ether. Combining the first and second crops gave **15** (62.02 g, 90%) as a white crystalline solid: mp 118–120 °C; $[\alpha]^{21}_D$ −18.7°; R_f(B) 0.90; ^{13}C NMR δ 193.9, Pac CO; 172.7, Lys CO; 172.1, Pro CO; 166.0, HMB CO; 157.0, Fmoc CO; 156.4, Boc CO; 144.8, Fmoc Cq; 142.7, HMB C1; 141.6, Fmoc Cq; 134.8, HMB C4; 134.6, Pac C1; 130.2, HMB C2; 129.7, Pac C2; 129.4, HMB C3; 128.5, Pac C3; 128.4, HMB C4; 128.3, Fmoc Ct; 127.7, Fmoc Ct; 125.8, Fmoc Ct; 120.8, Fmoc Ct; 78.3, Boc Ct; 67.4, Pac CH_2; 65.4, Fmoc CH_2; 65.3, CH_2; 58.7, Lys Cα; 52.0, Pro Cα; 46.8, Pro Cδ; 46.5, Fmoc CH; 30.2, Pro Cβ; 28.9, Lys Cβ; 28.4, Lys Cδ; 28.1, Boc CH_3; 24.6, Pro Cγ; 22.3, Lys Cδ. Lys Cϵ obscured by solvent.

Anal. Calcd for $C_{47}H_{51}N_3O_{10}$: C, 69.01; H, 6.29; N, 5.14. Found: C, 69.07; H, 6.39; N, 5.18.

4-[[[N^{α}-(*tert*-Butoxycarbonyl)-N^{ϵ}-(9-fluorenylmethoxycarbonyl)-L-lysyl-L-prolyl]oxy]methyl]benzoic Acid Hemihydrate (16). Zinc powder (25 g, 382 mmol) was added to a suspension of **15** (43.00 g, 53 mmol) in EtOAc (120 mL), AcOH (300 mL), and water (120 mL). The mixture was stirred for 16 h. The solution was filtered, the zinc was rinsed with MeOH (50 mL), and the combined filtrate was evaporated at 50 °C in vacuo. The resulting solid was partitioned between EtOAc (300 mL) and hot water (300 mL). The organic phase was washed sequentially with 10% citric acid/brine (1:1) and brine and dried. Evaporation of the solution afforded a gum, which was mixed with ether (100 mL). The product was precipitated by addition of petrol (100 mL). The precipitated oil was left to stand under ether/petrol (1:1) (200 mL) for 3 days, after which time the product had crystallized. The solid was washed with ether (50 mL) and air dried, to furnish **16** (33.14 g, 89%) as a white powder; mp 104–107 °C; $[\alpha]^{21}_D$ −31.2°; R_f(A) 0.83, R_f(B) 0.64; ^{13}C NMR δ 172.7, Lys CO; 172.1, Pro CO; 168.1, HMB CO; 157.0, Fmoc CO; 156.4, Boc CO; 144.8, Fmoc Cq; 141.7, HMB C1; 141.6, Fmoc Cq; 131.1, HMB C4; 130.1, HMB C1; 128.3, HMB C3; 128.2, Fmoc Ct; 127.7, Fmoc Ct; 125.8, Fmoc Ct; 120.7, Fmoc Ct; 78.5, Boc Ct; 65.4, Fmoc CH_2 and HMB CH_2; 58.7, Pro Cα; 52.0, Lys Cα; 46.8, Pro Cδ; 46.4, Fmoc CH; 30.2, Pro Cβ; 28.9, Lys Cβ; 28.4, Lys Cδ; 28.1, Boc CH_3; 24.6, Pro Cδ; 22.3, Lys Cγ. Lys Cϵ obscured by solvent.

Anal. Calcd for $C_{39}H_{45}N_3O_9 \cdot 0.5H_2O$: C, 66.08; H, 6.53; N, 5.93. Found: C, 65.97; H, 6.50; N, 5.75.

***N*-(2,4-Dinitrophenyl)-β-alanine (22).** A solution of FDNB (13.00 g, 70 mmol) in dioxane (100 mL) was added to a stirring solution of βAla (5.921 g, 66 mmol) in 10% aqueous Na_2CO_3 (170 mL). After 18 h, the solution was acidified with 10% citric acid and the product extracted with EtOAc (3 × 200 mL). The combined organic phase was washed with 10% citric acid/brine (1:1), then brine, and dried. The solid obtained upon evaporation was triturated with ether (100 mL) to afford yellow crystals of **22** (14.72 g, 87%): mp 145–147 °C; R_f(A) 0.41.

Anal. Calcd for $C_9H_9N_3O_6$: C, 42.36; H, 3.55; N, 16.47. Found: C, 42.30; H, 3.54; N, 16.28.

Preparation of Pins for Peptide Synthesis. Preparation of the polyethylene pins up to the stage ready for peptide synthesis is described elsewhere.[9] Kits containing functionalized pins (cleavable or noncleavable) are available for research purposes from Chiron Mimotopes Pty., Ltd., or Cambridge Research Biochemicals Ltd., Cheshire, United Kingdom.

Polyacrylic acid grafted pins[9] were coupled with (*tert*-butoxycarbonyl)-1,6-diaminohexane and Boc deprotected. A limited amount of Fmoc-βAla-OH was coupled, the unreacted amine on the pin surface was then capped by acetylation, and the Fmoc protection removed. Typically a βAla loading of 50 to 100 nmol per pin was used. Linkers were incorporated onto the pins at this point. Where peptide synthesis was performed, a second βAla residue was coupled to the Lys side chain to act as a spacer between the target peptide and the linker.

Peptide Synthesis. Peptides **17–21** were synthesized using N^{α}-Fmoc protected amino acids. Amino acids requiring side-chain deprotection were as follows: Arg(Pmc), Asp(OBu^t), Glu(OBu^t), His(Trt), Lys(Boc), Ser(Bu^t), Thr(Bu^t), Tyr(Bu^t). All peptide coupling reactions were performed in polypropylene microtitre plates using 150 μL of the activated amino acid solution per pin. The coupling solutions contained the desired Fmoc-protected amino acid (30 mM), HOBt (60 mM), and DCC (33 mM) in DMF. Couplings were performed for 16 h at 25 °C. Following a 5-min wash with MeOH and air drying, the pins were Fmoc deprotected by treatment with 20% piperidine in DMF (100 mL per 96 pins) for 30 min. Deprotection was followed by a DMF wash, two MeOH washes, and then air drying. This process was continued until the target peptides were assembled. Peptides were then acetylated using Ac_2O/Et_3N/DMF (5:1:50, v/v/v). Peptides were side-chain deprotected by treatment with TFA/phenol/EDT (95:2.5:2.5, v/w/v) for 5 h at 20 °C. Following air drying and vacuum desiccation for 1 h, 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 0.1 M pH 3 sodium phosphate–citrate buffer (100 mL per 96 pins) for 5 h. Peptides were then cleaved from the solid support by treatment with 0.1 M pH 7 sodium phosphate buffer (150 μL per pin) in the wells of a microtitre plate for 3 h at 20 °C.

Model Stability and Cleavage Studies. Model systems **7** and **8** were prepared by coupling **22** to pins derivatized with **5** and **6**, respectively. Boc deprotection was effected using TFA (30 min). Sonication and precleavage buffer soaks were as described above. All cleavages were performed at 20 °C using 150 μL of the test solution per well. The degree of cleavage was assessed by determining the absorbance of the cleavage solution

at 405 nm using a Titretek Multiscan Mk310. The amount of Dnp-containing peptide (X) per well was calculated using

$$X = [37.53 \times (\text{absorbance}) - 0.38] \text{ nmol}$$

Peptide Appraisal. Analytical HPLC was performed on a Waters Associates liquid chromatography system comprised of two 510 pumps, a WISP 710B autosampler, a Model 440 UV detector (254 nm) with an extended wavelength module (214 nm), and a DEC Professional work station. A 5-μm Merck Lichrosphere 100 RP-18 (250 × 4 mm i.d.) column was used. Gradient elution from A to B was carried out in 5–20 min with the following buffer system: A, 0.1% TFA in water, and B, 0.1% TFA in water/acetonitrile (40:60 v/v).

Amino acid analysis was carried out using PITC derivatives[20] as previously reported.[13] Positive ion FAB mass spectra were recorded by Dr. D. P. Kelly and Mr. B. Shirriffs, University of Melbourne. Samples were suspended in a thioglycerol matrix and ionized by bombardment with xenon ions.

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Biosynthesis of Sarubicin A. Synthesis and Incorporation of 6-Hydroxy[$^{13}CO^{15}NH_2$]anthranilamide[1]

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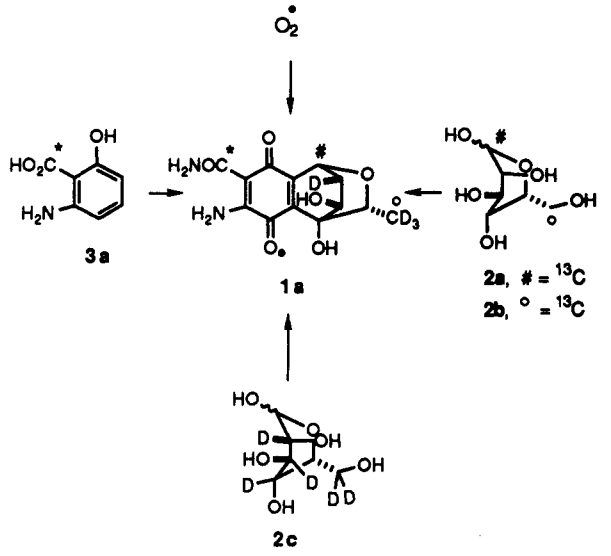
We have previously demonstrated that 6-hydroxyanthranilic acid (**3**) is specifically incorporated into sarubicin A (**1**) by *Streptomyces helicus*. 6-Hydroxyanthranilamide (**4**) has now been synthesized in five steps from *m*-anisidine in a manner that allowed efficient introduction of isotope labels to prepare [$^{13}CO^{15}NH_2$]-**4a**. A new synthesis of **3** from *m*-anisidine has also been developed. **4a** was fed to *S. helicus*, and a 1.29% incorporation into **1b** was obtained. Examination of the ^{13}C NMR spectrum of **1b** revealed predominantly intact incorporation, with a minor amount of **4a** (0.13%) first undergoing in vivo hydrolysis to the corresponding acid. Thus, carboxamide formation is the next step in the biosynthesis of **1**.

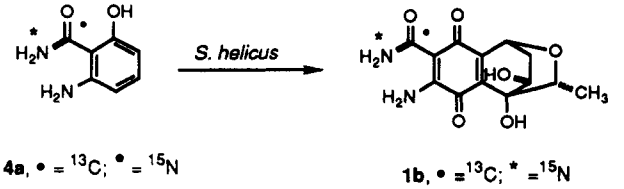
Sarubicin A (**1**), a quinone antibiotic produced by *Streptomyces helicus*, was first characterized in 1980[2] and its absolute stereochemistry established in 1983.[3] Two total syntheses have since been reported.[4] Early, unpublished results by a group at The Upjohn Company established the labeling pattern from [1-^{13}C]-D-glucose (**2a**) and [6-^{13}C]-D-glucose (**2b**), which indicated intact incorporation of glucose into the tetrahydropyran portion of **1**[5] and suggested a derivation of the quinonoid portion of **1** from the shikimate pathway.[6]

We subsequently established that [$^{13}COOH$]-6-hydroxyanthranilic acid (**3a**) labeled the carboxamide carbon of **1** and that the C-4 quinone carbonyl oxygen, but not that at C-1, was derived from $^{18}O_2$.[7] 6-Hydroxyanthranilic acid had not previously been known as a natural product. Results obtained from the incorporation of [2,3,4,6,6-$^2H_5$]-D-glucose (**2c**) showed retention of deuterium at the C-7_{cis} and methyl hydrogens in a 1:3 ratio, indicating replacement of the C-2 hydroxyl by hydrogen (retention of configuration) and migration of the C-4 deuterium to C-6 of glucose.[8] These results are summarized in Scheme I with the composite structure **1a**.

Scheme I



Scheme II



It was anticipated that subsequent key steps in the biosynthetic pathway leading to **1** would be formation of a *C*-glycoside, introduction of the C-6 phenol, cyclization, and generation of the carboxamide. In order to test whether carboxamide formation might be the first of these, 6-hydroxyanthranilamide labeled with both ^{13}C and ^{15}N

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