4 Results and Discussion

4.1 Attempted approaches towards synthesis of a cyclic depsipeptide

The project was divided into three phases.

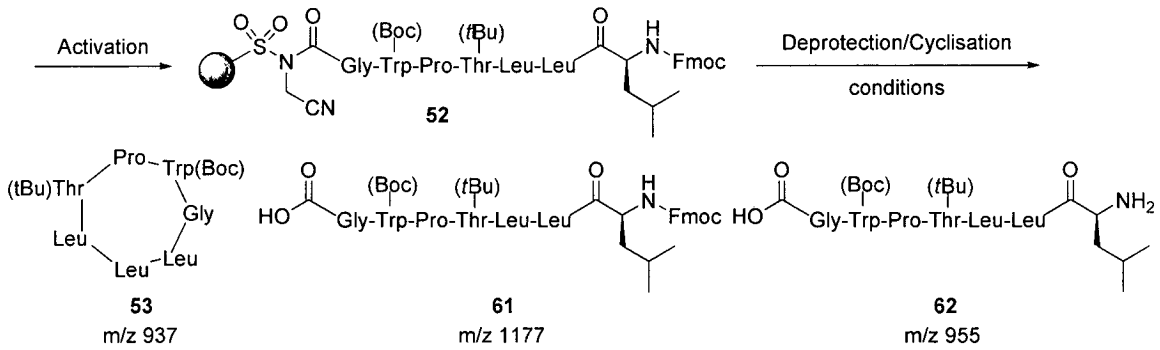
4.1.1 Phase I: A model study – synthesis of a cyclic peptide

Phase I: The desired linear peptide (**51**) was synthesised manually by coupling the first amino acid with solid-supported safety-catch linker **5**. Fmoc-Gly was chosen as the first residue to avoid any potential racemisation during the first coupling. Addition of the first amino acid as a mixture of 4 eq of Fmoc-protected amino acid, 4 eq of HBTU and 8 eq of DIPEA in DMF to the resin was carried out twice to ensure attachment. Minimum reaction time of 30 min was applied by agitating the reaction vessel for each coupling experiment. Unreacted free amino termini were capped with acetic anhydride (a solution of 4 eq of acetic anhydride, 4 eq of HOBt and catalytic amount of DMAP in DMF). Experimental resin substitution was calculated, based on UV absorption of the Fmoc group removed, to quantify attachment of the first residue. The Fmoc protecting group was removed, prior to addition of the following residue, using 20% piperidine in DMF for 30 min. A mixture of Fmoc-Trp(Boc) was added to couple with the free amine terminal of glycine as the second residue. Deprotection and coupling were repeated with corresponding Fmoc-protected amino acids (Fmoc-Pro, Fmoc-Thr(*t*Bu), and three consecutive units of Fmoc-Leu) until peptide **51** was achieved. Each coupling and Fmoc deprotection step was assessed by ninhydrin tests. However, amino acid deletions, which had certain residues missing in the final peptide, were observed during mass spectrometry analysis. This problem was successfully solved by increasing the reaction time for each coupling step to at least 1 h.

The safety-catch linker of peptide **51** (the sulfonamide part) was then activated overnight using iodoacetonitrile (20 eq) in the presence of DIPEA (10 eq) in NMP. It is worthy of note that it was necessary for iodoacetonitrile to be freshly filtered through a plug of basic alumina prior to use and the reaction carried out in the dark due to the photosensitivity of the reagent. The requisite activated linear peptide **52** was achieved, to be used in the following cyclisation studies.

The cyclisation studies were centred on finding suitable reaction conditions that removed the Fmoc group from **51** as well as assisted intramolecular lactamisation to afford cyclic peptide **53** in a one-pot fashion. Each cyclisation experiment was performed on a 10-mg scale of the activated resin **52**. The Fmoc group was first removed under basic conditions. Results from these cyclisation studies are illustrated in Table 1. The use of non-nucleophilic 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base provided more of the desired cyclic product than

what DIPEA did (Entries 5 vs. 6). In order to encourage cyclisation, it was found that heat, either conventional or microwave, helped promote the intramolecular peptide bond formation to afford **53**. However, the occurrence of **61** and **62** as by-products suggested that water was a major competitive nucleophile to the terminal free amine group in attacking the electrophilic carbonyl carbon (Scheme 14). This process took place as the peptide was cleaved from the resin. Means to get rid of water from the reaction were applied, including the use of microwave heating in an open system to allow water to escape below the boiling point of the solvent used (Entries 14-15), and the use of 4Å molecular sieves under anhydrous conditions (Entries 16-20). Pleasingly, yields were found to increase as more water was eliminated. Moreover, sufficient reaction time was deemed important to fully remove the Fmoc group and encourage cyclisation (Entries 17-19). All relevant peptides were identified and yields of **53** obtained by ESI-HRMS (Bruker Daltonics, Bellerica, MA, U.S.A.).



Scheme 14 Cyclisation studies

Table 1 Results of the cyclisation studies

Entry	Activation	Conditions	Temp	Time	Results
1	ICH_2CN, DIPEA, DMF	2% DBU/THF	rt	O/N	peptide still on resin
2	ICH_2CN, DIPEA, DMF	DIPEA/DMF	rt	O/N	peptide still on resin
3	ICH_2CN, DIPEA, DMF	10% thiophenol/DMF	rt	24 h	no cyclisation
4	ICH_2CN (filtered), DIPEA, DMF	2% DBU/THF	rt	30 min	v. small amount of product (937), mainly linear (955) and linear Fmoc (1177), some deletions

5	ICH_2CN (filtered), DIPEA, DMF	DIPEA/THF	rt	O/N	free amine on resin, nothing in solution
6	ICH_2CN (filtered), DIPEA, DMF	2% DBU/THF	rt	O/N	v. small amount of (937), mainly (955) and (1177)
7	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF MW, 30PSI, 200W	100°C	30 min	small amount of (937), mainly (955) and (1177); but better than previous sets of conditions
8	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF MW, 30PSI, 200W	rt	30 min	small amount of (937), mainly (955) and (1177)
9	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF MW, 30PSI, 200W	200°C	30 min	small amount of (937), mainly (955) and (1177); but poorer than Entry 7
10	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF conventional heat	100°C	30 min	v. small amount of (937), mainly (955) and (1177)
A new batch of peptide-loaded resin synthesised with longer reaction times					No deletions observed
11	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF 4Å MS	rt	30 min	major improvement observed by HPLC, better conversion 15%
12	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF 4Å MS	70°C	30 min	18%
13	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF 4Å MS	100°C	30 min	16%
14	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF MW (open)	70°C	30 min	37%
15	ICH_2CN (filtered), DIPEA, DMF	2% DBU/DMF MW (open)	100°C	30 min	32%
16	ICH_2CN (filtered), DIPEA, DMF	excess DBU/DMF (anhydrous) (bead) 4Å MS	100°C	30 min	37%
17	ICH_2CN (filtered),	excess	100°C	30 min	36%

	DIPEA, DMF	DBU/DMF (anhydrous) powder 4Å MS			
18	ICH_2CN (filtered), DIPEA, DMF	excess DBU/DMF (anhydrous) powder 4Å MS	100°C	3 h	56%
19	ICH_2CN (filtered), DIPEA, DMF	excess DBU/DMF (anhydrous) powder 4Å MS	100°C	25 h	40% with no (1177) observed
20	ICH_2CN (filtered), DIPEA, DMF	excess DBU/DMF (anhydrous) powder 4Å MS Resin dreid *in vacuo* for 1 h	100°C	3 h	34%

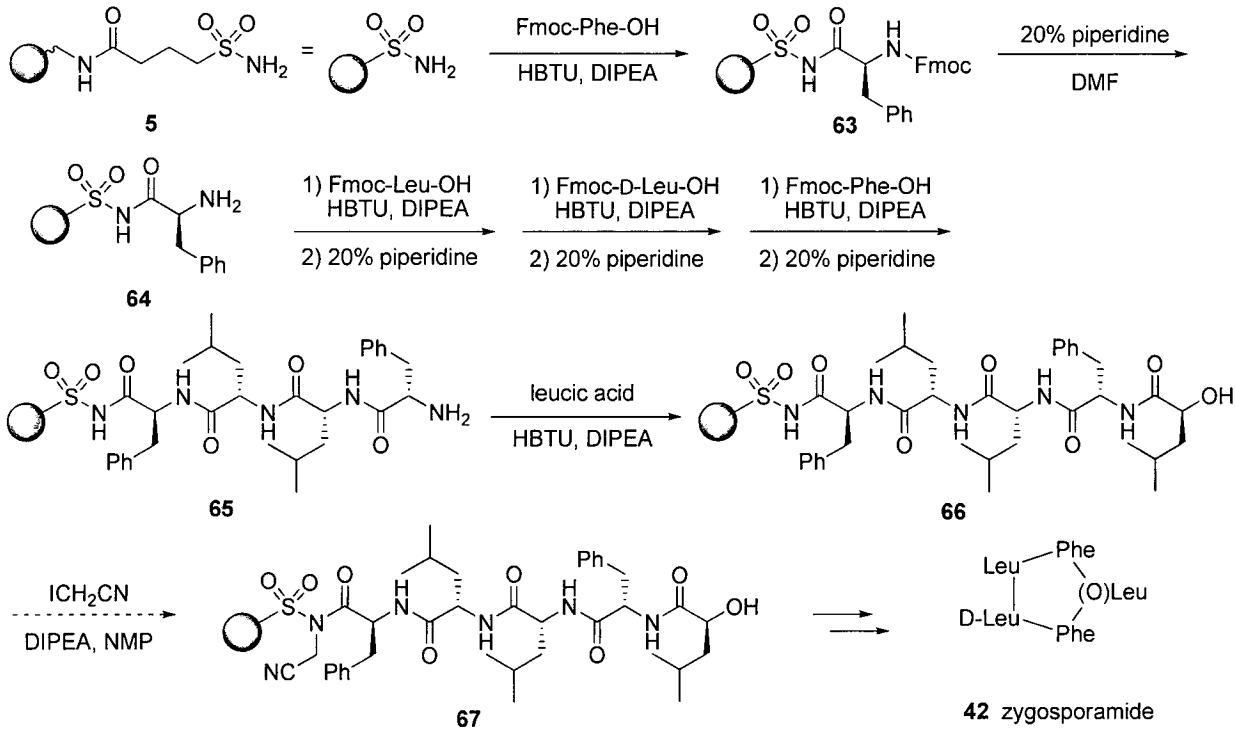
From the results shown above, it could be suggested that water was problematic, at the crucial cyclisation step, as a major competitive nucleophile. However, it supported the proposed macrocyclisation idea to achieve intramolecular ester bond formation (Phase II). Under these circumstances, an *O*-nucleophile seemed a more active nucleophile than its *N*-counterpart to work as our desired ring closing transformation.

Suitable gradient reversed-phase HPLC systems to isolate the compounds produced from the cyclisation experiments were investigated. Initial conditions of 0-100% MeCN in H_2O were used. Collected portions of eluates were submitted to ESI-HRMS (Bruker Daltonics, Bellerica, MA, U.S.A.) to identify relevant peptides. Reversed-phase HPLC purification was repeated with MeCN being replaced by isopropanol (IPA) from initial conditions of 0-100% IPA to 20-100%, 40-100%, 50-100% and 40-90% IPA depending on polarity of peptides involved (**53**, **61** and **62**). Once the cyclic product (**53**) was isolated, its amino acid side-chains were globally deprotected by treating with Reagent K (82.5% TFA, 5% thioanisole, 5% m-cresol, 5% water, and 2.5% ethanedithiol) for 4 h to achieve the requisite natural product integerrimide A (**40**). Cold methyl-*tert*-buthyl ether (MTBE, cooled at -20°C over 6 h) was added to precipitate the peptide product, which would be isolated by decanting off the solvents. It was found that the amount of peptide **53** obtained was so small that precipitation was difficult. The resulting product was, instead, purified by reversed-

phase HPLC, where initial conditions of 0-60% MeCN were applied. The isolated amount of compound **53** was submitted for ^{1}H NMR spectroscopy for further confirmation. However, more material was required to fully characterise using other techniques. Generation of sufficient material was left to be carried out when time permitted.

4.1.2 Phase II: Attempted development of a method to synthesise a cyclic depsipeptide

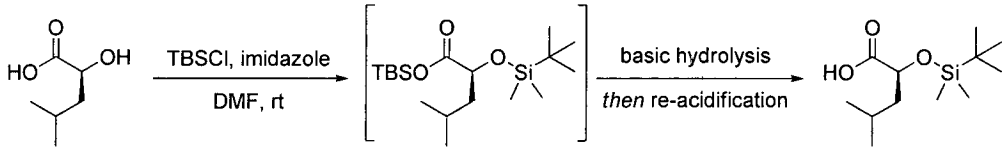
Phase II: It was decided to move on to studying cyclisation of a depsipeptide with terminal *O*-nucleophile without further optimising results from Phase I. The requisite linear peptide **55** (from Scheme 13) was prepared using the same protocol as that in Phase I. Unprotected leucic acid was first investigated to provide **66** (Scheme 15). Ninhydrin tests were used to monitor each deprotection and coupling step. However, it was found that coupling of the last residue, leucic acid, gave a different ninhydrin result, where some colourless resins changed back to deep blue over a short period of time. This coupling step was therefore left to react much longer. A ninhydrin test was carried out again after 24 h of reaction, but the colour change was still different from the usual cases (the presence of a free $-NH_2$: deep blue; the absence: colourless). However, it is precedented that α-hydroxy acids can couple with another amino acid or a peptide chain as well as an amino acid does. A new batch of **66** was then prepared without the coupling time of the final residue being extended.



Scheme 15 Studies towards the synthesis of zygosporamide

One of the original intentions for this phase was also to investigate whether a protecting group was required for the α-hydroxy acid to couple and cyclisation to take place. Theoretically, the hydroxy group can be left free during coupling. However, high potential for the free hydroxy group to react with the activating agent, iodoacetonitrile, can be expected under the same activation conditions (rt, overnight) as the intended unit of the safety-catch linker does. If certain reaction conditions were to be designed to achieve selectivity, means to determine yields would be difficult for solid-supported substrates and the correctly activated resins impossible to separate from the rest. It was, hence, decided that protection of the α-hydroxy acid should be established prior to coupling.

Silyl protecting groups seemed an obvious choice for protection of a hydroxy group or an alcohol. *tert*-Butyldimethylsilyl chloride (TBSCl) was chosen due to its availability. Usually, a strategy would involve, first, protecting the more chemoselective carboxylic acid unit and then the hydroxy group before liberating the carboxylic acid unit again. The desired product would eventually be obtained after the first protecting group was removed. In order to reduce steps, at least 2 eq of TBSCl could be employed to ensure bis-protection of both active sites. The resulting reaction mixture would then be subjected to hydrolysis, and then re-acidification to neutralise the carboxylic acid back in the organic phase (Scheme 16). Since the hydrolysis and re-acidification steps would be performed as part of work-up, this could shorten the length of the synthesis without having to isolate the intermediates.



68 69 70

Scheme 16 *O*-Protection strategy

There are a few TBS protection protocols available in literature (Table 2). Conditions reported by Menche and co-workers[76] were, in fact, applied from those established by Ley *et al.* with different equivalents of reagents used.[77, 78] However, it was chosen to follow those reported by Menche *et al.* first as the reaction was carried out at room temperature, which was more practically convenient.

Table 2 Literature procedures for TBS protection

Authors	Conditions	Quenching	Hydrolysis/Acidification	Yield
Masuda *et al.* 2002[79]	3 eq TBSCl 4 eq imidazole DMF, rt, 4 h	quench: H_2O extract: ether wash: H_2O, brine	dilute: THF acidify: 0.1M HCl, stir: 4 h, rt	80%

Ley *et al.* 2003[77, 78]	3.8 eq TBSCl 2.0 eq imidazole DMF, 0 °C, 22 h	dilute: 1:1 EtOAc/petrol wash: citric acid, H_2O	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	80%
Menche *et al.* 2009[76]	2.4 eq TBSCl 4.8 eq imidazole DMF, rt, O/N	dilute: 1:1 EtOAc/toluene wash: citric acid, sat. $NaHCO_3$, H_2O, brine	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	74%
PhD thesis (serine) 2008*	1 eq TBSCl 10 eq imidazole DMF, rt, 30 min then 60 °C, 24 h	conc.	dilute: petrol extract: 5% $NaHCO_3$ wash: petrol acidify to pH 3 (1M $KHSO_4$)	73%

It was found that the desired product could not be prepared by following the procedure reported by Menche *et al.*,[76] nor the starting material recovered (Table 3, Entry 1). Washing with a saturated solution of $NaHCO_3$ prior to hydrolysis-acidification seemed to be the cause. It is also worth noting that the reaction mixture in the procedure reported by Ley and co-workers was worked up without this additional basic washing (Table 2).[77] After this step was removed, more promising results were obtained (Table 3, Entry 4). The differences between the Menche and Ley methods were solely during the reaction itself: amounts of reagents used, reaction temperatures and times (Table 2, Entries 2-3). An alternative workup protocol, which was found successful in the TBS protection of serine (Table 2, Entry 4),* was followed in hopes of improving matters (Table 3, Entries 2-3, 5-8 and 11). It, unfortunately, gave poorer results, where the products obtained were identified to be the leucic acid starting material. It was suspected that the desired TBS protection might have actually taken place but was, by some means, removed under the reaction conditions. The workup procedure employing K_2CO_3 was therefore chosen as the hydrolysis step of choice (Table 3, Entries 9-10 and 12-14).

More reasonable reaction times of 2-4 h provided a better yield (9:1, **70/68**) than leaving it to react for much longer (24 h, 4:1) (Entries 4 vs. 10). Moreover, heating was found unnecessary for this transformation, where the reaction could proceed at room temperature (Entries 13 vs. 14). It

* S. Kumarn, PhD thesis, 2008, University of Cambridge, Cambridge, UK.

was also noticed that the reaction time for basic hydrolysis of 6 h was found to give a significantly better result than that of 4 h, used in reported procedures (Entries 10 vs. 13).

Table 3 TBS-protection studies

Entry	Conditions	1st step workup	Hydrolysis/Acidification	Results
1	2.4 eq TBSCl 4.8 eq imidazole DMF, rt, 24 h	dilute: 1:1 EtOAc/toluene wash: citric acid, sat. $NaHCO_3$, H_2O, brine conc. TLC: TBS off O/N	re-dissolve: MeOH K_2CO_3, H_2O, 14 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	TBS-OH
2	2.4 eq TBSCl 4.8 eq imidazole DMF, rt, 23 h	dilute: hexane TLC: TBS off O/N	extract: 5% $NaHCO_3$ wash: hexane 0 °C, acidify to pH 4 (10% citric acid)	S.M. - incorrect int. for Si-CH_3 - O<u>H</u> present - δ 4.27
3	2.4 eq TBSCl 4.8 eq imidazole DMF, rt, 20 h	dilute: hexane TLC: TBS off O/N	extract: 5% $NaHCO_3$ wash: hexane 0 °C, acidify to pH 4 (1M $KHSO_4$)	S.M. - incorrect int. for Si-CH_3 - O<u>H</u> present - δ 4.27
4	3.4 eq TBSCl 5.9 eq imidazole DMF, rt, 23 h	dilute: 1:1 EtOAc/hexane wash: citric acid, H_2O, brine conc. TLC: OK	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	mixture - 4:1 (δ 4.27:4.17) - int. for Si-C<u>H</u>$_3$ not 1/5 but 2.6/5 - likely be S.M. (with v. br O<u>H</u>)
5	2.8 eq TBSCl 5.3 eq imidazole DMF, rt, 24 h	conc. TLC: good	dissolve: hexane extract: 5% $NaHCO_3$ wash: hexane 0 °C, acidify to pH 4 (1M $KHSO_4$)	S.M. - pH 6: δ 4.17; incorrect int. for Si-C<u>H</u>$_3$; O<u>H</u> present - pH 4: δ 4.27; no S C<u>H</u>$_3$; O<u>H</u> present

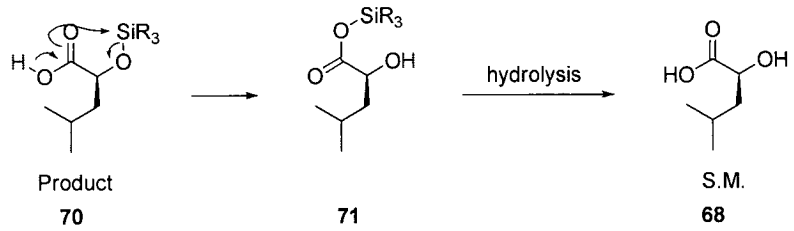
6	3.6 eq TBSCl 6.8 eq imidazole DMF, rt, 24 h	dilute: hexane TLC: TBS partially off after 3 h	extract: 5% $NaHCO_3$ 0 °C, acidify to pH 4 (10% citric acid)	S.M. - δ 4.17; no Si-C<u>H</u>3; O<u>H</u> present
7	2.9 eq TBSCl 5.7 eq imidazole DMF, rt, 2.5 h	dilute: hexane TLC: good	extract: 5% $NaHCO_3$ wash: hexane 0 °C, acidify to pH 4 (10% citric acid)	S.M. - δ 4.17; no Si-C<u>H</u>3; O<u>H</u> present
8	3.0 eq TBSCl 5.6 eq imidazole DMF, 60 °C, 2.5 h	dilute: hexane conc. TLC: good	dissolve: hexane extract: 5% $NaHCO_3$ 0 °C, acidify to pH 4 (10% citric acid)	S.M. - incorrect int. for Si-C<u>H</u>3 - O<u>H</u> present - δ 4.20
9	3.5 eq TBSCl 6.0 eq imidazole DMF, rt, 5.5 h	dilute: 1:1 EtOAc/hexane wash: H_2O, brine conc.; kept in fridge O/N TLC: good	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	S.M. - incorrect int. for Si-C<u>H</u>3 - O<u>H</u> present - δ 4.27
10	3.2 eq TBSCl 5.7 eq imidazole DMF, rt, 3.5 h	dilute: 1:1 EtOAc/hexane wash: H_2O, brine conc. TLC: good	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc.; kept in fridge O/N dilute: H_2O 0 °C, acidify to acc pH 4 (10% citric acid)	mixture - 9:1 (δ 4.27:4.17) - int. for Si-C<u>H</u>3 not 1/10 but 6/10 - likely be product (with a v. br peak)
11	3.2 eq TBSCl 5.7 eq imidazole DMF, rt, 3.5 h	dilute: 1:1 EtOAc/hexane TLC: good	extract: 5% $NaHCO_3$ 0 °C, acidify to acc pH 4 (10% citric acid)	TBS-OH
12	3.3 eq TBSCl 6.4 eq imidazole DMF, 60 °C, 3 h	dilute: 1:1 EtOAc/hexane wash: H_2O, brine conc.; kept in fridge O/N TLC: TBS off	re-dissolve: MeOH K_2CO_3, H_2O, 4 h then conc. dilute: H_2O 0 °C, acidify to acc pH 4 (10% citric acid)	TBS-OH
13	3.3 eq TBSCl 5.8 eq imidazole DMF, rt, 4 h	dilute: 1:1 EtOAc/hexane wash: H_2O, brine	re-dissolve: MeOH K_2CO_3, H_2O, 6 h then conc. dilute: H_2O	mixture - 97:3 (δ 4.27:4.17) - int. for Si-C<u>H</u>3 not

		conc., kept in fridge O/N TLC: good	0 °C, acidify to pH 4 (10% citric acid)	0.97 but 0.82 - likely be product (with a v. br peak)
14	3.3 eq TBSCl 5.9 eq imidazole DMF, 60 °C, 3.5 h	dilute: 1:1 EtOAc/hexane wash: H_2O, brine conc., kept in fridge O/N TLC: TBS partially off after 3 h	re-dissolve: MeOH K_2CO_3, H_2O, 6 h then conc. dilute: H_2O 0 °C, acidify to pH 4 (10% citric acid)	mixture - 97:3 (δ 4.27:4.17) - int. for Si-C<u>H</u>$_3$ not 0.97 but 0.82 - likely be product
15	3.2 eq TBSCl 6.0 eq imidazole DMF, rt, 4 h	dilute: 1:1 EtOAc/hexane wash: H_2O, conc. TLC: good		mixture - 23.5:76.5 (δ 4.50:4.35) - No δ 0.12, hence no product
16	3.3 eq TBSCl 5.5 eq imidazole DMF, 60 °C, 2 h	dilute: 1:1 EtOAc/hexane wash: H_2O, conc. TLC: TBS partially off after 2 h		mixture - 1:2 (δ 4.28:4.15) - complicated Si-C<u>H</u> area
17	3.6 eq TBSCl 6.3 eq imidazole DMF, rt, 26 h	dilute: 1:1 EtOAc/hexane wash: H_2O, conc. TLC: good		mixture - NMR 3 weeks afte - 3:2 (δ 4.23:4.12) - product-like preser

Bis-TBS protection reactions, without the hydrolysis-acidification workup, were carried out to compare (Table 3, Entreis 15-17). Although no product was observed in the reaction mixtures after intended reaction times were completed, it was found that the crude products changed over time. These observations were made using ^{1}H NMR spectroscopy. The TBS group seemed to have fallen off from either the hydroxy or carboxylic sites, where a product-like structure (**70**) was identified after the material was kept for 3 weeks. However, after 3 months, it was identified to be the structure of the free leucic acid (**68**). NMR samples (in $CDCl_3$) were combined with their corresponding crude products from the bis-TBS protection reactions prior to *in vacuo* concentration and stored in a -4 °C refrigerator. It could be suggested that contact with the acidic $CDCl_3$ NMR solvent might promote TBS removal. Additional NMR studies to monitor stability of

the bis-TBS product should, therefore, be carried out without combining NMR samples back and that using C_6D_6 as a sole solvent for comparison.

It can be seen that the carbonyl group at the alpha position affects the stability of the desired product (**70**) as the silyl group can, unfortunately, "walk" from the hydroxy group to the carbonyl neighbour (Scheme 17). After hydrolysis, this gives back the starting material (**31**). It is also worth noting that pKa of the resulting intermediate (**34**) was much larger than that in the desired product (**33**), suggesting that this process is not reversible.



Product
70

71

S.M.
68

Scheme 17 α-Carbonyl functional group nearby affecting the protected hydroxyl group

More conditions were investigated, but results were still unfruitful. The close vicinity of the two functional groups, namely carbonyl and hydroxyl, made this problem inevitable for Si-containing protecting groups. Although it was planned to investigate into alternative protection strategies, including benzyl (Bn), allyl and para-methoxybenzyl (PMB) groups, lengthy steps would be involved just to obtain the terminal residue of the requisite peptide chain. Further studies of the actual proposal concept of deprotection and on-resin cyclisation to give the cyclic depsipeptide natural product must also follow. It could be foreseen that an extensive amount of time would be required to achieve this research aim. In order to get performed results published, a new direction of the project to focus on cyclic peptides instead of cyclic depsipeptides was considered.

4.2 A new direction of the project: synthesis of a cyclic peptide natural product

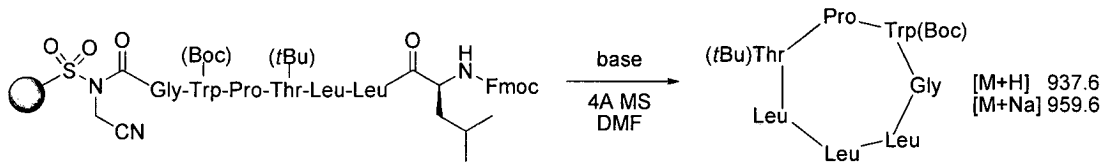
The solid-phase peptide synthesis to provide linear peptide **51**, a requisite intermediate in synthesis of integerrimide A, has been investigated in Phase I (Scheme 12). Since in-detail and more extensive studies of this work reserve their own merit of publication due to the new tandem approach planned during the deprotection–macrocyclisation steps. Hence, it was decided to divert the direction of the project to development of a method to synthesise cyclic peptides and its application in natural product synthesis. A target compound chosen for the new direction to investigate the methodology should be a cyclic peptide natural product with decent bioactivity and whose synthesis has no previous reports so that, once the method could be developed, the synthesis itself could be reported as a total synthesis.

4.2.1 Methodology development and optimisation studies

Relatively large reactions (approximately 500 mg of resin **5** was used for each reaction vessel) to prepare a sufficient amount of linear peptide **51** were carried out. The reaction time of 3 h was allowed for each amino acid coupling step and 30 min still for Fmoc deprotection using 20% piperidine in DMF. The resulting linear peptide (**51**) was then subjected to activation of the safety-catch linker to obtain **52** ready for subsequent cyclisation studies.

Loaded resin **52** (approximately 5 mg for each reaction) was subjected to various reaction conditions to remove the terminal Fmoc group to liberate the free amine, which should subsequently undergo nucleophilic displacement at the carbonyl carbon of the first amino acid, right next to the activated linker, for the desired cyclic peptide to be formed as well as to leave the solid support into solution at the same time. The first batch of reactions was simply reproduction of reaction conditions attempted previously as Phase I but with different analysis techniques (Table 4). Originally, crude reaction mixtures after intended reaction times were liquid chromatographed (HPLC) and each fraction collected was subjected to high-resolution mass spectrometry (HRMS) due to unavailability of a liquid chromatography-mass spectrometer (LCMS) at the time of studies (in Phase I). Moreover, various reaction conditions for DBU as a base were the main concentration then. Therefore, this new batch of reactions involved many other bases as candidates for the Fmoc removal and each experiment was analysed by LCMS. Since they were the first set of reactions to be analysed by LCMS, careful studies of the resulting chromatographic profiles were carried out.

Table 4 Results obtained for the first batch of cyclisation reactions



52 53

Entry	Base	Temp (°C)	Time	Results ($\times 10^6$ counts)
1	DBU	rt	2 h	610
2	Phosphazene	rt	1.5 h	293
3	TEA	rt	1.5 h	273
4	Cs_2CO_3	rt	1 h	398

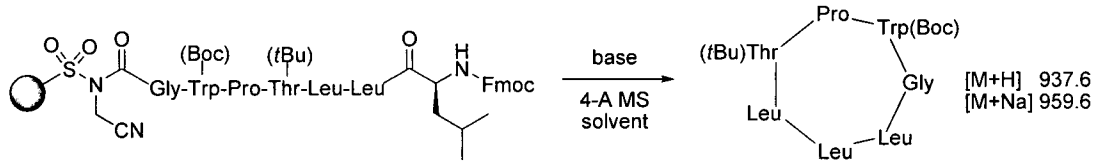
5	K_2CO_3	rt	1.5 h	75
6	DIPEA	rt	1 h	6
7	DBU	70	1 h	342
8	Cs_2CO_3	70	1 h	394
9	K_2CO_3	70	1 h	763
10	DMAP	rt	1 h	380
11	K_2CO_3	0	1 h	115
12	DMAP	0	1 h	116
13	DMAP	70	1 h	282
14	K_2CO_3 + DMAP	rt	1 h	323

Work-up of each reaction involved filtering a crude reaction mixture (approximately 1 ml in volume) through a 22-µm pore-sized syringe filter and washing with acetonitrile (1 ml of MeCN, 4 more times) to dilute. Each LCMS sample was prepared by further diluting such solution 10-fold to obtain a 50-fold dilute sample for LCMS analysis. The required mass for cyclic peptide **53** was m/z 937 ($[M+H]^+$); however, m/z 959, which corresponded to $[M+Na]^+$ was also observed. The numbers of counts reported in Table 4 are sums of areas under curves of both m/z 937 and 959 (peak at 36 min; LCMS: 0-100% MeCN with 0.1% formic acid in H_2O with 0.1% formic acid, 40 min, flow rate 0.2 ml/min, on an Acclaim® Dionex 120A C18 column, 3 µm, 150 × 2.1 mm). From Table 4, it was found that the reaction employing potassium carbonate (K_2CO_3) at 70 °C for 1 h was promising (entry 9). However, another set of reactions was required to repeat these findings as well as to ensure consistency of the work-up procedure used as some reactions were left at room temperature for some time before being worked up. Interestingly, a small amount of a dimer of the cyclic peptide (14-mer cyclic peptide) was observed (m/z 937 for $[M+H]^{2+}$). Such finding was more frequent when DBU was used as a base than that with K_2CO_3 or TEA.

The second batch of reactions involved repeats of conditions in the first batch but with a more proper work-up protocol and uniform reaction times as well as a number of other conditions employing different bases (Table 5). For more standardised dilution procedure, the volume of 5 ml of the total solution of each crude reaction mixture after filtration and MeCN washings was ensured, before the subsequent 10-fold dilution, using a 5-ml volumetric flask for accurate

comparison of numbers of counts obtained by LCMS. Moreover, an additional cyclic peptide[†] (5 ppm) was added to each LCMS sample to verify reliability and reproducibility of the protocol used on LCMS (0-100% MeCN with 0.1% formic acid in H_2O with 0.1% formic acid, 40 min) and the LCMS instrument itself. However, it is worth noting that the results were originally recorded as numbers of counts obtained from areas under peaks by LCMS, and %yields were later calculated based on the manufacturer's stated resin substitution using a calibration curve constructed in an LCMS quantitative study, which is covered in detail in Section 4.2.2.

Table 5 Results obtained for the second batch of optimisation reactions in cyclisation studies[a]



52 53

Entry	Base	Solvent	Temp (°C)	Time	Results (10^6 counts)	% Yield[b]
1	DBU	DMF	rt	1 h	282.0	9.33
2	DIPEA	DMF	rt	1 h	142.6	4.72
3	Na_2CO_3	DMF	rt	1 h	295.7	9.78
4	K_2CO_3	DMF	rt	1 h	310.4	10.27
5	Cs_2CO_3	DMF	rt	1 h	233.4	7.78
6	Phosphazene[c]	DMF	rt	1 h	205.4	7.04
7	TEA	DMF	rt	1 h	335.5	11.10
8	DMAP	DMF	rt	1 h	66.2	2.19
9	K_2CO_3+DMAP	DMF	rt	1 h	224.8	7.47
10	Pyridine	DMF	rt	1 h	264.7	8.76
11	DBU	DMF	70	1 h	98.4	3.26
12	K_2CO_3	DMF	70	1 h	94.1	4.41
13	Cs_2CO_3	DMF	70	1 h	127.4	4.43
14	TEA	DMF	70	1 h	211.1	8.87
15	DMAP	DMF	70	1 h	125.4	4.22
16	K_2CO_3+DMAP	DMF	70	1 h	108.6	3.66
17	DBU	DMF	0	1 h	60.6	2.00
18	K_2CO_3	DMF	0	1 h	5.5	0.19
19	DMAP	DMF	0	1 h	16.0	0.54

[†] A generous donation by Dr Arthit Makarasen

20	Na_2CO_3	DMF	rt	1 d	764.7	25.29
21	Na_2CO_3	DMF	rt	7 d	720.8	23.84
22	K_2CO_3	DMF	rt	1 d	267.7	9.01
23	K_2CO_3	DMF	rt	3 d	354.5	11.73
24	K_2CO_3	DMF	rt	5 d	351.3	11.62
25	K_2CO_3	DMF	rt	7 d	337.9	11.38
26	K_2CO_3	NMP	rt	1 d	207.3	6.74
27	K_2CO_3	NMP	rt	3 d	296.4	9.81
28	K_2CO_3	NMP	rt	5 d	289.5	9.58
29	K_2CO_3	NMP	rt	7 d	264.8	8.76
30	DIPEA	DMF	rt	1 d	581.1	19.22
31	Pyridine	DMF	rt	1 d	264.5	8.75
32	DBU	DMF	rt	1 d	104.7	3.46
33	DBU	DMF	rt	3 d	121.5	4.09
34	TEA	DMF	rt	1 d	766.5	25.32
35	TEA	DMF	rt	3 d	781.2	25.84
36	TEA	DMF	rt	7 d	942.3 (773.8)	31.17 (25.64)[d]
37	TEA	DMF	rt	14 d	1184.3 (1496.8)	39.17 (49.52)[d]
38	TEA	DMF	rt	1 h	149.9	4.96[e]

[a] General conditions: loaded resin **52** (approx. 5.6 mg, 2.43×10^{-6} (theoretical) mol), 4-Å MS (approx. 10 mg) and base (10 eq) in a solvent (1 ml). [b] Overall yield of 15 steps determined by LCMS analysis as the number of counts of ions $[M+H]^+$ and $[M+Na]^+$ combined, which were later converted to concentration, and then % yield, from the slope of a calibration curve generated using **53** itself as a standard ($y=6.5351\times10^7x$).[‡ c] P_4-*t*-Bu in *n*-hexane. [d] On a scale 100 folds larger than the general conditions. [e] The reaction was sonicated for 1 h, using ice to maintain the temperature of the ultrasonic bath.

It is worth nothing that the numbers of counts reported from LCMS were later quantified into chemical yields after a calibration curve of a suitable internal standard was constructed (Section 4.2.2). In the meantime, a cyclic peptide reference compound kindly provided by a colleague[§] was added as an internal reference to verify reliability and reproducibility of the LCMS protocol used.

Each number of counts reported in **T**able 5 is an average of two consecutive runs on LCMS. The numbers of counts for the constant amount (5 ppm) of the cyclic peptide added were found to

[‡] A quantitative LCMS study conducted by Mr Nitirat Chimnoi

[§] Dr Arthit Makarasen

vary from 483.0 to 1,582.0×10^6 counts when already prepared LCMS solvent mixtures were used. The matter of inconsistency was investigated to find that the cyclic compound used as an internal reference was unstable under the LCMS conditions, rendering it not suitable as an internal standard for our system. It was also found that the mobile phases (MeCN with 0.1% formic acid and H_2O with 0.1% formic acid) needed to be freshly prepared each day to make the numbers of counts of the peptide internal reference comparable (900 – 1,100×10^6 counts). Nevertheless, when freshly prepared solvent mixtures were used, it verified the reliability and reproducibility of the analysis procedure used to quantify the desired product.

From Table 5, a number of bases were screened at room temperature for 1 h (entries 1-10). K_2CO_3 and TEA were found to be comparably high yielding (entries 4 *vs.* 7). Additional variations to the reaction temperature were investigated at 70 and 0 °C to represent higher and lower temperatures than the convenient room temperature, respectively, for a selected number of bases (entries 11-16 and 17-19). It was found that none gave any better yield that its corresponding entry of the same base at room temperature. This may be explained that lower temperatures, albeit suitable for solution-phase macrocyclisation such as the Yamaguchi esterification, did not provide sufficient energy to overcome energy barriers required for this solid-phase situation. On the other hand, the raised temperature of 70 °C may have destroyed any product formed *in situ* or provided too much entropy for the compound to be constrained into a ring. Furthermore, an alternative solvent, NMP, was investigated to compare with DMF using K_2CO_3 as a base for various reaction times of 1 h, 24 h, 3 and 7 days (entries 22-25 *vs.* 26-29). However, it was not found to provide any better results than the usual solvent, DMF. The optimum reaction time was investigated using the base of choice, TEA, (entries 34-37). Although the conditions employing TEA in DMF at rt for 14 days were found to give the best yield (entry 37), the difference from that of 7 days was not proportional, suggesting that the reaction time of 7 days was optimal (entry 36). A means of sonication was investigated in hopes of shortening the reaction time but did not give a fruitful result (entry 38), even though care was taken to control the reaction temperature.

Interestingly, careful analysis of the LCMS chromatograms showed that in many cases the area under curve of m/z 959 was greater than that of m/z 937, suggesting that the cyclic peptide formed tended to trap Na^+ whose traces may have contaminated the reaction or LCMS system somehow. The m/z that corresponded to $[M+K]^+$ and $[M+Cs]^+$ were also looked into in reactions employing K_2CO_3 and Cs_2CO_3 as a base but their areas under curves were found to be very small compared with that of $[M+Na]^+$. Such information provoked ideas to further investigate the relationship between the peptide chain lengths, hence ring sizes, and the presence of suitable cations, which may influence the macrocyclisation by pre-organisation of the peptide chain to ease or encourage the ring closure. Another base, which could also provide sodium ions, namely Na_2CO_3, was also investigated (entries 3 and 20-21). Unfortunately, it was not found to be better

than TEA. Nevertheless, such ideas of peptide chain pre-organisation may still be further studied by varying peptide chain lengths, hence cyclic peptide ring sizes, as possible future work.

4.2.2 Synthesis of integerrimide A natural product

Although it is known in literature that the presence of proline, which is considered a turn-inducer, in a given linear peptide can encourage macrocyclisation to take place by pre-organising the linear peptide in solution so that both ends are in close proximity,[67-70] cyclisation of such a peptide with only three to eight amino acids is still usually very difficult, especially on-resin.[24, 71, 72] Apart from being generally low yielding,[66] such difficulty may possibly lead to epimerisation at the *C*-terminal amino acid and, particularly in solid-phase synthesis, formation of cyclic dimers and oligomers.[67, 68] Moreover, proline-containing cyclic peptides often exist as mixtures of conformers because the *cis*- and *trans*-peptide bonds of a proline residue are restricted to interconversion by conformational constraints. Together with its reported biological activity[73] and potential in further structure-activity relationship studies, these make the synthesis of integerrimide A a challenge in synthetic chemistry.

As outlined in Scheme 12, we envisioned our synthesis plan to involve macrocyclisation between glycine (Gly) and leucine (Leu) so that Gly, which lacks stereogenic centres, would be the first residue and proline (Pro) in the middle of the sequence in order to provide a strong turn-inducing element to facilitate effective cyclisation.[67] Moreover, in solid-phase peptide synthesis (SPPS), it is precedented that having proline[80] or tryptophan[81, 82] as the first residue can result in the formation of a diketopiperazine causing the dipeptide to leave the solid support. Although standard solid-phase peptide synthesis from *C*- to *N*-termini is reported to offer minimal racemisation,[83] Gly was still chosen as the first amino acid for coupling with the solid-supported safety-catch linker. This was to avoid any chance of racemisation the first coupling might cause as it is usually carried out twice to ensure maximum loading. Furthermore, it was envisaged that the terminal free amine would more readily attack a less sterically hindered electrophilic carbonyl during the crucial macrocyclisation step.

Synthesis of the requisite linear peptide sequence (**51**) began with loading Gly onto the 4-sulfamylbutyryl AM resin (**5**), whose reported loading procedure involved the use of PyBOP and *N*,*N*-diisopropylethylamine (DIPEA) in DMF, or $CHCl_3$ in the case of other Fmoc-amino acids than Gly, first at –20 °C for 8 h and then at room temperature (rt) overnight.[36] Since racemisation was not an issue for Gly, loading efficiencies of the PyBOP conditions and the standard Fmoc protocol utilising HBTU were compared (Table 6). It was found by Fmoc removal UV analysis that complete loading was obtained after two repetitions using the standard HBTU conditions (entry 4). Although the Gly loading efficiency obtained with PyBOP was found to be lower than the

literature value (entry 1; 32% *cf.* 89% yield),[36] it agreed with that reported by Andrews *et al.* for the same residue (0.3 mmol/g).[66] Loading efficiency was determined by UV analysis of the Fmoc group removed into solution upon treating dried resin **49** (1-2 mg) with 20% piperidine in DMF (1 ml) for 30 min using a UV-visible spectrophotometer at fixed wavelengths of 301, 302 and 304 nm. Each sample was prepared by diluting 250 μl of its corresponding reaction mixture with DMF to reach a volume of 5 ml (volumetric flask). The sample volume of 3 ml was held in a 4-ml cuvette for analysis. A solvent blank (3 ml), from 150 μl of 20% piperidine in DMF and 2850 μL of DMF, was used as a reference.

$$loading = \frac{Abs \times 20}{\varepsilon^{301} \times m^{resin}}$$

The above formula was used to acquire glycine loading efficiency in $mol \cdot g^{-1}$, where Abs is an average absorption of 10 scans measured relative to the solvent blank recorded at 301 nm; 20 is the dilution factor; ε^{301} is 7800, which is a known molar extinction coefficient for a dibenzofulvene adduct (the Fmoc fragment removed into solution) at 301 nm;[84] m^{resin} is mass of dried resin **49** used in mg.

Table 6 First loading of Fmoc-Gly-OH onto resin **5**

Entry	Conditions	1st coupling	2nd coupling	% Yield[a]
1	Fmoc-Gly-OH (4 eq), DIPEA (6 eq), PyBOP (3 eq), DMF, rt	8 h, then O/N[b]	-	32
2	Fmoc-Gly-OH (4 eq), DIPEA (6 eq), PyBOP (3 eq), DMF, rt	8 h, then O/N[b]	O/N	49
3	Fmoc-Gly-OH (4 eq), DIPEA (8 eq), HBTU (4 eq), DMF, rt	3 h	-	56
4	Fmoc-Gly-OH (4 eq), DIPEA (8 eq), HBTU (4 eq), DMF, rt	3 h	O/N	quant.[c]
5	Fmoc-Gly-OH (4 eq), DIPEA (8 eq), HBTU (4 eq), DMF, rt	8 h	-	71
6	Fmoc-Gly-OH (4 eq), DIPEA (8 eq), HBTU (4 eq), DMF, rt	8 h	O/N	78[c]

[a] Yields determined by UV spectroscopy after Fmoc removal, based on the manufacturer's stated resin substitution of 0.90 mmol/g. [b] The reaction was carried out at –20 °C for 8 h, then rt overnight. [c] Yield for a double coupling, followed by capping.

The remaining structure of linear peptide **51** was assembled by standard Fmoc solid-phase peptide synthesis[85] and cyclisation using the optimal reaction conditions developed. The reaction was scaled up to prepare cyclic peptide **53** enough for full characterisation studies as well as to be used as an internal standard to construct a calibration curve for the LCMS system so that the reported numbers of counts could be quantified into chemical yields.

The scaled up reaction to prepare **53** was carried out 100 times larger than that of each optimisation experiment. The number of counts corresponding to the sum of m/s 937 and 959 was pleasingly comparable with its small-scale counterpart for LCMS samples of the same dilution (for 14 days, 1,184.3 *vs.* $1{,}496.8\times10^6$ counts for small and large scales, respectively). A greater number of counts in the larger experiment might correspond to systematic errors being better overcome. The rest of the crude reaction mixture was filtered, first, through a pad of Celite and washed with MeCN, and then through 22-μm pore-sized syringe filters and washed with MeCN. The resulting filtrate was concentrated to remove MeCN and as much DMF as possible. The concentrated crude product was subjected to preparative HPLC analysis using a reverse-phase C18 column (Grace Vydac 218TP1022 Protein & Peptide C18) with the same conditions but with an adjusted flow rate proportional to those performed on an analytical column (ana: 0-100% MeCN, flow 0.5 ml/min, 40 min; prep: 0-100% MeCN, flow 11 ml/min, 40 min). Collected fractions were analysed using LCMS or HRMS when available. It was found that fractions collected around a retention time of 36 min still corresponded to m/z 937 and 959. To accommodate the entire volume of the crude product per maximum loading of the column, preparative HPLC analysis was divided into several runs, each with an injection of 400-900 μl. Fractions containing m/z 937 and 959 were be combined and further purified using a smaller gradient analysis (50-70% MeCN, flow 11 ml/min, 40 min). After cyclic peptide **53** was isolated, it was subjected to quantitative LCMS analysis to construct a calibration curve as self-comparison would be more accurate.

While the $[M+H]^+$ ions at *m/z* 955.6, which corresponded to linear peptide **72** formed from competing nucleophilic attack of water at the *C*-terminus, were often observed, albeit minimal in cases using TEA as a base, none of the $[M+H]^+$ ions at *m/z* 994.6 and 1033.6 corresponding to possible linear peptides **73** and **74**, respectively, (resulting from replacement of Fmoc on the *N*-terminus with the $-CH_2CN$ group from iodoacetonitrile) were detected during LCMS analysis (Figure 5). However, a minute amount of $[M+H]^+$ ions at *m/z* 1038.6, which may be due to the Fmoc group being partially destroyed in the presence of iodoacetonitrile (**75**), was co-eluted with other mass-to-charge ratios at 29 min (LCMS: 0-100% MeCN with 0.1% formic acid in H_2O with 0.1% formic acid, 40 min, flow rate 0.2 ml/min, on an Acclaim® Dionex 120A C18 column, 3 μm, 150 × 2.1 mm). In each optimisation experiment, the intensity of such mass-to-charge ratio was

so small, compared with that of **53** at 36 min, that it was considered negligible. Furthermore, the $[M+Na]^+$ ions at *m/z* 1060.6 detected by high-resolution mass spectrometry (HRMS) in eluates from preparative reversed-phase high-performance liquid chromatography (RP-HPLC) of scale-up experiments (0-100% MeCN in H_2O, 40 min, flow rate 11 ml/min, on a Vydac® 218TP™ C18 column, 10 µm, 250 × 22 mm) did not correspond to **75**. Pleasingly, this suggested that the replacement of Fmoc with an acid-labile protecting group was not necessary.

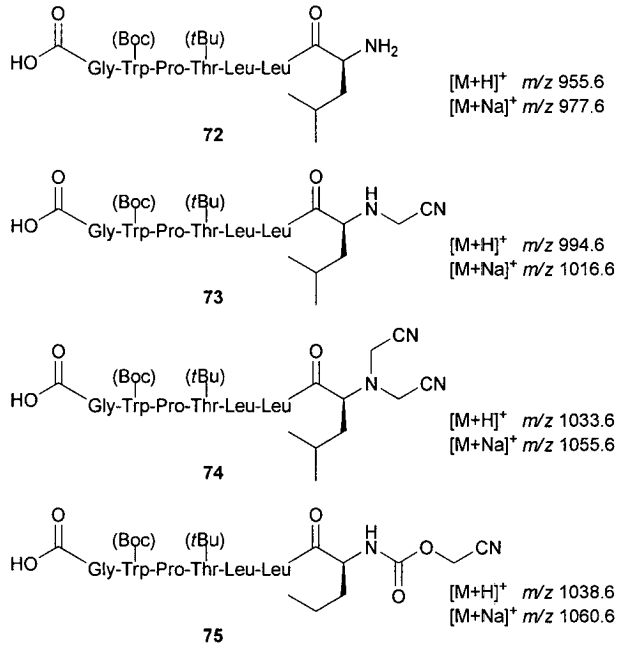
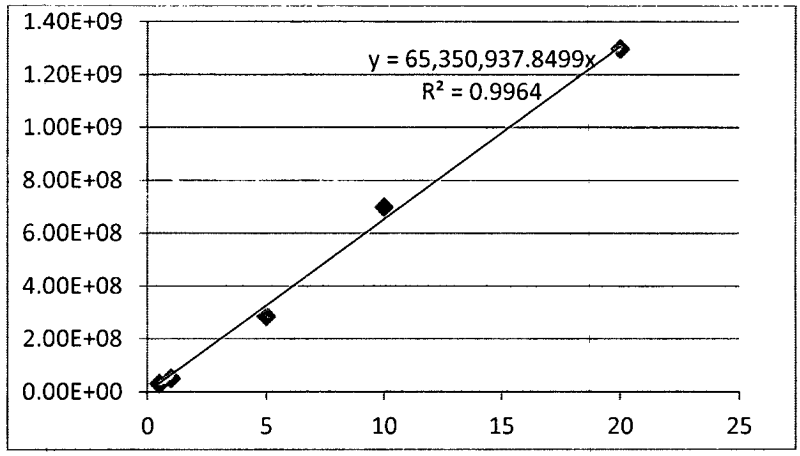


Figure 5 Possible linear peptides from **52** in the presence of iodoacetonitrile.

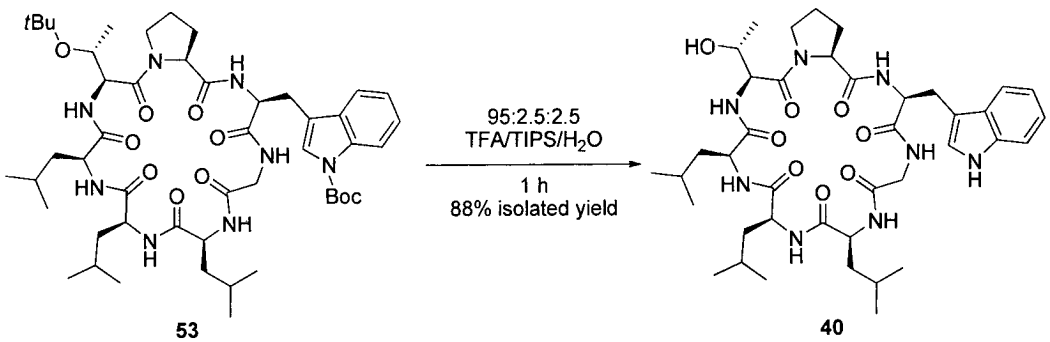
The quantitative LCMS analysis was studied at concentrations 0.5, 1, 5, 10 and 20 mg/ml to plot a calibration cure, which resulted in a linear equation $y = 6.5351 \times 10^7 x$, where x = concentration and y = the number of counts of the sum of $[M+H]^+$ and $[M+Na]^+$ ions of **53** at *m/z* 937.6 and 959.6, respectively ($R^2 = 0.9964$; Scheme 18).



Scheme 18 The calibration curve obtained from quantitative LCMS analysis to convert numbers of counts to % yields

The slope of the linear equation was used to convert numbers of counts of **53** obtained from the optimisation studies (Table 5) to concentrations and then % yields based on the manufacturer's stated resin substitution which have already been included in Table 5 (the last column).

Cyclic product **53** was isolated by preparative RP-HPLC (peak at 36 min, 0-100% MeCN in H_2O, 40 min, flow rate 11 ml/min, on a Vydac® 218TP™ C18 column, 10 μm, 250 × 22 mm), and further purified also by preparative RP-HPLC (peaks at 27 and 31 min, 50-70% MeCN in H_2O, 40 min, flow rate 11 ml/min). Interestingly, it was found that other HPLC systems of either higher or lower gradients, including isocratic, did not give as good a separation for the same flow rate. Ratios of the areas under curves of the two peaks at 27 and 31 min also varied depending on concentrations of the loaded samples. HRMS revealed the same mass-to-charge ratio ($[M+Na]^+$ at *m/z* 959.6) for both eluates. However, the $[M+Na]^{2+}$ ions at *m/z* 959.6 which corresponded to the cyclic dimer of **53** were also detected at extremely low intensities in the 31-min fraction. Upon separate re-injections to the same HPLC system, both 27- and 31-min fractions were eluted at 31 min, delightfully suggesting that they were concentration-dependent conformers rather than epimers. Eventually, **53** prepared under the optimal reaction conditions of 7 d was isolated by preparative RP-HPLC in 21% overall yield over 15 steps, based on the manufacturer's stated resin substitution of 0.90 mmol/g.Suitable cleavage cocktails to remove side-chain protecting groups of Trp-containing peptides include Reagents B (88:5:5:2 TFA/phenol/water/TIPS) and R (90:5:3:2 TFA/thioanisole/EDT/anisole). However, simple, non-odorous mixtures are largely used.[86] The 95:2.5:2.5 TFA/water/TIPS mixture was therefore chosen to carry out global side-chain deprotection of **53** to achieve the desired natural product (**40**) (Scheme 19). A set of experiments were conducted for 1, 2, 3, 4, 5 h and overnight to find that 1 h was the most suitable reaction time as the starting material was completely consumed at 1 h and the product from reduction of the indole unit in Trp to indoline started to be seen by LCMS from 2 h onwards.[87] The material was brought through to obtain integerrimide A in 88% isolated yield after two sets of preparative RP-HPLC. It is worth noting that the two product fractions at 23 and 28 min from the first system (0-100% MeCN in H_2O, 40 min, flow rate 11 ml/min, on a Vydac® 218TP™ C18 column, 10 μm, 250 × 22 mm) were concentrated and further purified separately in the second system (10-70% MeCN in H_2O, 40 min, flow rate 11 ml/min, on a Vydac® 218TP™ C18 column, 10 μm, 250 × 22 mm) to give the desired product at a single retention time of 32 min with no corresponding cyclic dimer observed. The second system was performed as small amounts of oxidised derivatives started to be observed and increased over time around the 28-min peak eluded by the first system.



53
$[M+H]^+$ *m/z* 937.6
$[M+Na]^+$ *m/z* 959.6

40
$[M+H]^+$ *m/z* 781.5
$[M+Na]^+$ *m/z* 803.4

Scheme 19 Global side-chain deprotection of **53** to achieve **40**

We were pleased to find that the spectral characteristics of the resulting cyclic peptide **40** was identical in all respects to those of natural integerrimide A, except the ratio of conformers exhibiting *cis*- and *trans*-Pro amide bonds in ^{1}H NMR spectra recorded in DMSO-d_6 which was believed to be due to the difference in concentration (2.4:1, *cf.* 4:1 in lit.).[73] Particularly, the specific optical rotation of synthetic **40** was found to match that of the natural isolate, suggesting that all amino acids constituting the natural product are of their natural L-form indicating the absolute stereochemistry of **40** as drawn in Scheme 19 and that this synthesis caused no epimerisation. It is also noteworthy that integerrimide A was oxidised when left as a solution of DMSO-d_6 at room temperature for 7 d. The route described herein to synthesise integerrimide A may also be used to allow access to a library of its analogues for structure-activity relationship studies.