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COMMUNICATION

Synthesis of Endolides A and B; Naturally Occurring *N*-Methylated Cyclic Tetrapeptides†

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Abstract

Endolides A and B are naturally occurring, *N*-methylated, cyclic tetrapeptides possessing an unusual 3-(3-furyl)alanine amino acid and outstanding biological profiles. 1-Propanephosphonic anhydride (T3P) was used to mediate a solution-phase cyclisation reaction of the linear tetrapeptides, thus achieving the first syntheses of both endolides A and B. The stereoselectivity of the tetrapeptide cyclisation reactions was found to be reagent-controlled, and was independent of the *C*-terminal configuration of the linear peptide starting materials.

Introduction

Endolides A-D (**1**-**4**) are a small family of *N*-methylated cyclic tetrapeptides recently isolated from the marine sponge-derived fungus *Stachylidium* sp. 293 K04 (Figure 1).[1,2] Endolides A-D (**1**-**4**) possess a rare 3-(3-furyl)alanine amino acid, which is only reported to exist in two other distinct natural products; the heptapeptides rhizonins A and B,[3] and the cyclic pentapeptide bingchamide B.[4] While rhizonin A was prepared in 2009 using a solution-phase macrocyclisation of the linear heptapeptide,[5] rhizonin B and bingchamide B have yet to be synthesised. Both the rhizonins and bingchamide B were isolated from bacteria (*Burkholderia* sp., and *Streptomyces bingchenggensis* respectively), and as such, the 3-(3-furyl)alanine amino acid was thought to biosynthetically originate from bacteria.[2] This hypothesis is further supported by the recent discovery that *Burkholderia contaminans*, an endosymbiotic bacteria in *Stachylidium bicolor*, is in fact responsible for metabolism of the endolides.[6] X-ray crystallography of endolide A (**1**) showed the 3-(3-furyl)alanine residues possess an L-configuration, and that the cyclic tetrapeptide exists in a trans,cis,trans,cis (*tctc*) conformation.[1] The 3-(3-furyl)alanine residues in endolides B-D (**2**-**4**) were also assumed to possess an L-configuration based on biosynthetic considerations.[1,2]

Endolide A (**1**) was found to exhibit selective affinity for vasopressin receptor 1A (K_i = 7.04 μM), while endolide B (**2**) showed extremely selective affinity for the serotonin receptor $5HT_{2b}$ (K_i = 0.77 μM), with no affinity toward ten other serotonin receptor subtypes.[1] Selective antagonism of the $5HT_{2b}$ receptor reportedly enhances hepatocyte growth in models of acute and chronic liver injury, showing potential application in liver regeneration.[1] Endolides A and B (**1** and **2**) were found to be inactive in a vast array of other bioassays, including an absence of cytotoxicity against a panel of five cancer cell lines, thus demonstrating impressive target selectivity and a promising safety profile.[1]

While the cyclisation of tetrapeptides containing turn-inducing motifs (such as proline, D- or *N*-methyl amino acids) has been successful in several cases,[7–15] the cyclisation of all-L tetrapeptides is notoriously challenging due to geometric constraints of forming the cyclotetrapeptide.[16–18]

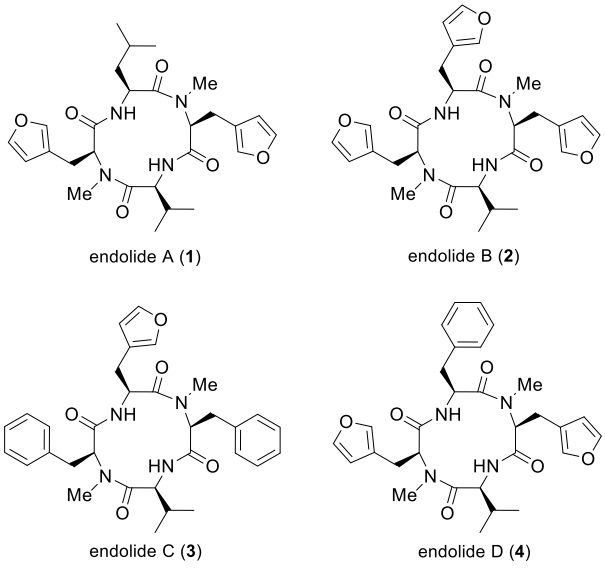

Figure 1 Endolides A-D (**1**-**4**)

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†Electronic Supplementary Information (ESI) available: Experimental procedures and analytical RP-HPLC chromatograms for all isolated peptides; ^1H, ^{13}C and HRMS spectra for compounds **1** and **2**; and complete spectroscopic data comparison of **1** and **2** with the isolation report. See DOI: 10.1039/x0xx00000x

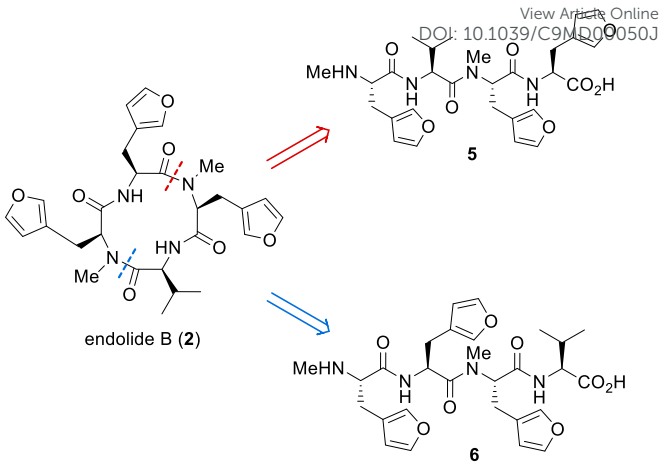
Tetrapeptides are also notoriously prone to *C*-terminal epimerisation during solution-phase cyclisations.[14,16,18] Heterochirality between termini due to the presence of an epimerised *C*-terminal D-amino acid frequently facilitates cyclisations of the peptides, since D-amino acids favour cyclisation-inducing cisoid conformations.[16,18] As such, cyclisations of the *C*-terminal epimers often experience enhanced reaction kinetics over their all-L counterparts.

Nevertheless, the attractiveness of cyclic tetrapeptides as pharmaceutical leads (due to their conformational rigidity and compliance with Lipinski's rules)[18] has driven recent research into their synthesis, and thus several useful reagents have been identified which have effectively cyclised all-L *N*-methyl tetrapeptides. Such reagents include 1-propanephosphonic anhydride (T3P) [as demonstrated by our recent syntheses of the *N*-methylated all-L cyclotetrapeptides onychocins A-D[14] and pseudoxyallemycin A (as yet unpublished)], and (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP).[19] However, unpublished results within our group suggest that the success of these reagents is sequence dependant, with preferential formation of the epimerised cyclic product occurring in several instances. Nevertheless, a desire to further probe the interesting biological activity of endolides A and B (**1** and **2**), prompted us to target their synthesis.

Results and Discussion

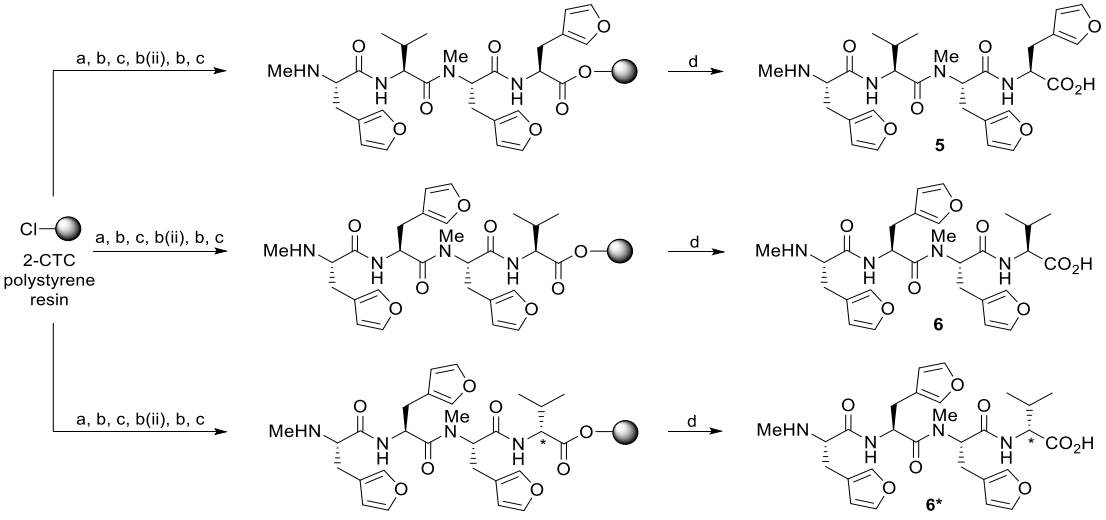
N-Methylated residues at the *N*-terminus of linear tetrapeptides were found to be the most effective precursors for solution-phase cyclisation during our previous syntheses of onychocins A-D, since they displayed reduced dimerization compared to their free amine counterparts.[14] As such, we chose to prepare both linear precursors **5** and **6** of endolide B (**2**) since tetrapeptide cyclisations are notoriously sequence dependant (Scheme 1).[14,20]

C-Terminal D-valine derivative **6*** was also desired (Scheme 2), to help elucidate whether epimerisation occurs during the cyclisation step; a common occurrence during solution-phase cyclisations.[14,16,18] Linear peptides **5**, **6** and **6*** were synthesised using Fmoc-solid phase peptide synthesis (SPPS) on 2-chlorotrityl chloride (2-CTC) resin using 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) as a coupling reagent, and 20% piperidine in DMF for removal of the Fmoc group (Scheme 2). Site-selective *N*-methylations were conducted on-resin in accordance with the procedure described by Kessler and co-workers.[21] Following resin-cleavage with 10% TFA in dichloromethane the linear tetrapeptides **5**, **6** and **6*** were purified by reverse-phase HPLC to facilitate monitoring of the subsequent cyclisation reaction by analytical HPLC and mass spectrometry (MS).



Scheme 1 Retrosynthetic analysis of endolide B (**2**).

Cyclisation was first attempted using PyAOP as the cyclisation reagent, due to its reported enhanced reaction kinetics over hydroxybenzotriazole (HOBt)-based reagents in peptide cyclisations.[19,22,23] Linear peptides **5** and **6** were treated with



Scheme 2 Synthesis of linear peptides **5**, **6**, and **6***. Reagents and conditions: a) Fmoc-AA-OH, DIPEA, DCM, rt, 1 h; b) Fmoc-SPPS (i) 20% piperidine in DMF, rt, 2 x 5 min; (ii) Fmoc-AA-OH, HATU, DIPEA, DMF, rt, 1 h; c) *N*-methylation (i) 20% piperidine in DMF, rt, 2 x 5 min; (ii) nosyl-Cl, *sym*-collidine, NMP, rt, 2 x 15 min; (iii) DBU, NMP, rt, 3 min *then* dimethyl sulfate, rt, 2 min; *repeat*; (iv) 2-mercaptoethanol, DBU, NMP, rt, 2 x 10 min; d) 10% TFA in DCM, rt, 10 min.
Fmoc-AA-OH = Fmoc-L-Val-OH, Fmoc-D-Val-OH or Fmoc-L-3-(3-furyl)Ala-OH[‡] as appropriate.

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PyAOP (3 equivalents) and DIPEA (6 equivalents) in DMF under high dilution (1 mM) at 40 °C (Scheme 3).

Monitoring of the cyclisation reactions of linear peptides **5** and **6** by analytical HPLC (Figure 2, red and blue traces respectively) showed formation of a common minor peak (retention time 25.2 min) with the desired mass of **2**, inferring that the desired product, endolide B (**2**), was being formed as a minor side product (3% and 5% respectively). However, the major products of the cyclisations of **5** and **6** possessed different retention times (22.3 min and 21.0 min respectively), but again, both with the same mass as that of cyclised endolide B (**2**) (analysed by RP-HPLC and MS). This indicated that these observed major products in the cyclisation of each of **5** and **6** correspond to the epimerised products **7** (55%) and **8** (33%) respectively. This result was disappointing, but not entirely surprising due to the propensity of *C*-terminal epimers to undergo cyclisation more readily than their all-L counterparts.[16,18]

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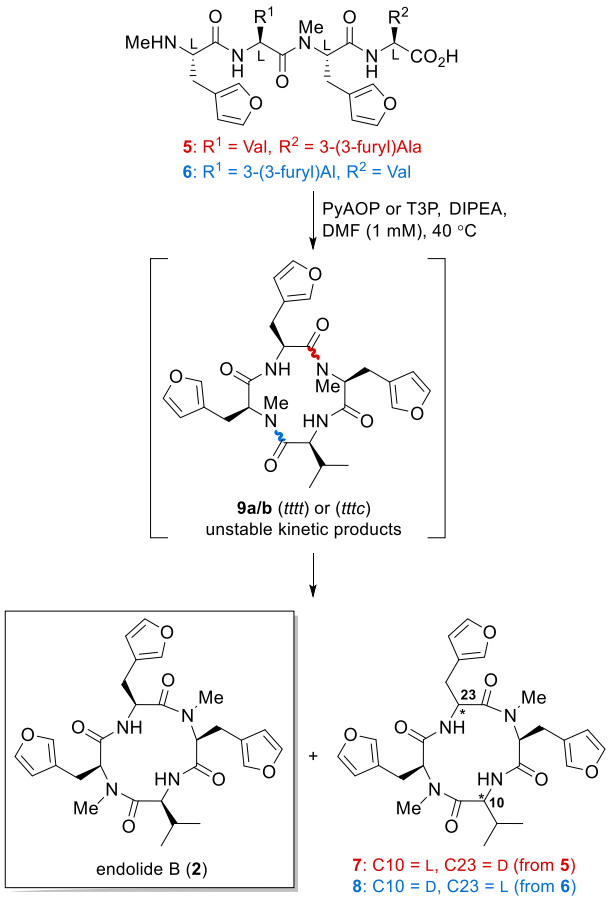
During our previous investigation into the cyclisation reactions of onychocins A-D, density functional theory (DFT) and NMR studies indicated that the cyclised kinetic products adopt an unstable all-trans (*tttt*) and trans-trans-trans-cis (*tttc*) configuration, which are both in slow exchange in solution.[14] The all-cis (*cccc*) and cis-cis-cis-trans (*ccct*) conformers were not considered given the known poor stability of these conformer in analogous cyclic systems.[14,24] The kinetic products were found to gradually convert to a thermodynamically stable *tctc* conformer, which, importantly, is the native conformation of endolide A (**1**).[1,14] In line with our previous work, close monitoring of the reactions showed rapid conversion (<10 min) of the linear precursors into the suspected kinetic products **9a**/**b** (t_R = 17.4 and 18.6 min from **5**, and t_R = 19.0 and 19.8 min from **6**) which were then converted to the thermodynamic cyclised products **7** or **8** (Scheme 3 and Figure 2, reaction monitoring).[14] The kinetic products **9a** and **9b** initially possessed a mass corresponding to that of cyclised endolide B (**2**) (analysed by RP-HPLC and MS). However upon purification by HPLC, and reanalysis of the fractions by MS, these kinetic products were found to have hydrolysed (with molecular weights matching those of the linear tetrapeptides **5** and **6**) after 6-24 hours in aqueous acetonitrile (with 0.1% TFA) at room temperature. This supported our proposed assignment of these kinetic products as the unstable, highly constrained *tttt* and *tttc* products **9a**/**b** since a similar observation was made during the synthesis of an onychocin analogue.[14] The aforementioned DFT calculations



Scheme 3 Products observed during the cyclisation of linear tetrapeptides **5** and **6**.

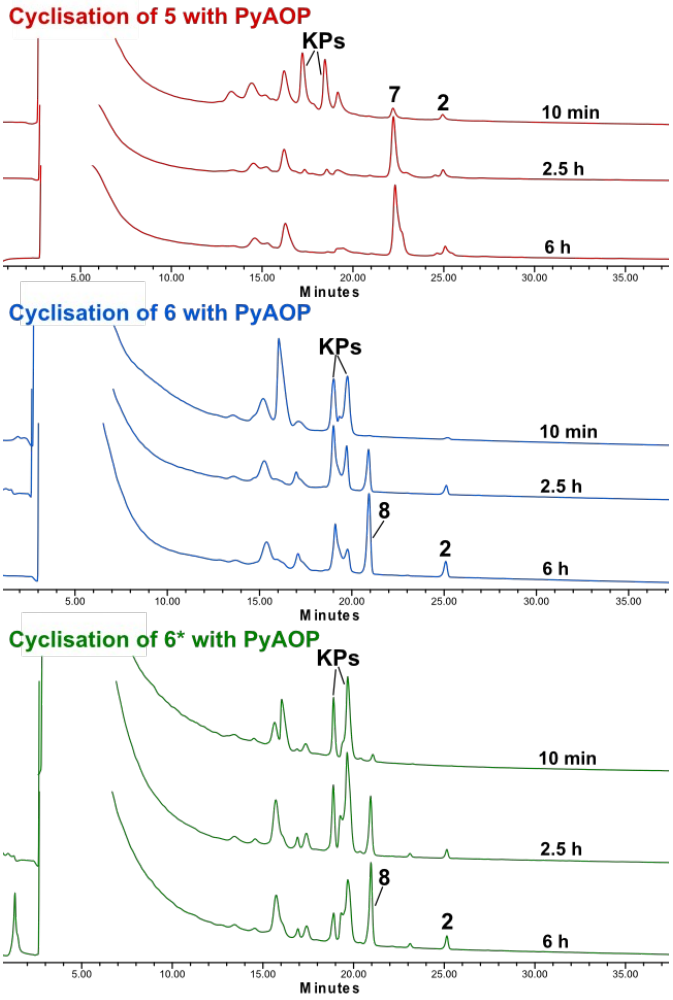


Figure 2 RP-HPLC (214 nm) profiles for the PyAOP mediated cyclisations of **5** (red, top), **6** (blue, middle) and **6*** (green, bottom) at 10 min, 2.5 h and 6 h. Linear gradient of 20–80% B over 40 min (~1% B min^{-1}) using a Luna C18 column (4.6 × 250 mm, 5 µm). A = 0.1% TFA in H_2O and B = 0.1% TFA in MeCN. KPs = kinetic products.

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conducted on the onychocin analogue conformers determined the stability of the conformers in solution to decrease in the order *tctc*>>*tttc*≥*tttt*.[14] It was assumed that a similar trend would hold for the endolide conformers, given their similar structures and experimental observations.

When linear peptide **6*** containing an unnatural *C*-terminal D-valine residue was subjected to the same reaction conditions, epimerisation was observed to occur far less readily than with the *C*-terminal L-valine peptide **6**, again affording **8** as the major product (26%), with minor epimerisation to yield endolide B (**2**) (3%) (Figure 2, green traces). This suggests that formation of endolide B (**2**) was disfavoured using PyAOP regardless of the stereochemistry of the *C*-terminal amino acid of the linear peptide precursor. A similar observation was recently made by Gunjal and Reddy during their attempted synthesis of the *N*-methylated cyclic tetrapeptide pseudoxyallemycin B, where 3-*epi*-pseudoxyallemycin was formed as the sole product during the T3P mediated cyclisation of linear precursors with both *C*-terminal L- and D-tyrosine residues.[16] The authors proposed that the selective formation of the undesired epimeric product could have resulted from introduction of a turn-inducing D-amino acid by *C*-terminal epimerisation and/or external conformational assistance of the activated T3P ester intermediate; both plausible explanations for this observed phenomenon. As such, it was thought that altering the coupling reagent, and hence the conformation of active ester intermediate, could facilitate cyclisation of the all-L linear peptides to provide endolide B (**2**).

To this end, we turned our attention to the use of T3P as a cyclisation reagent, since this has been reported to reduce the rate of epimerisation in difficult couplings.[14,25,26] Linear peptides **5** and **6** were subjected to the same conditions as used previously [1 mM in DMF with DIPEA (6 equivalents) at 40 °C] but with T3P as the cyclisation reagent (3 equivalents) (Scheme 3).

Pleasingly, monitoring of the reactions by analytical HPLC (after reaction times of 10 min, 5 hours and 23 hours) showed both linear peptides **5** and **6** formed a common major product, which possessed the same retention time (25.2 min) as that of the minor product formed using PyAOP (Figure 3, red and blue traces respectively). This was identified as the desired product, endolide B (**2**), following purification by preparative HPLC and MS and NMR analysis. Gratifyingly, the ^{1}H and ^{13}C NMR data of synthetic **2** was found to be in excellent agreement with the isolation report (see Tables S1 and S2 in the ESI).[1] The HPLC chromatograms of the cyclisation reaction profiles of **5** and **6** showed clean conversion of kinetic products **9a** and **9b** to the desired product **2**, with little formation of epimerised side products **7** and **8** respectively, albeit with reduced reaction kinetics (complete conversion after 23 hours). Excellent conversion was observed with peptide **6**, forming endolide B (**2**) in 91% purity, while conversion from peptide **5** was only 20% due to formation of additional unidentified side products.

As expected, when linear D-valine peptide **6*** was subjected to the same reaction conditions, slow epimerisation was observed to occur, affording **2** as the major product (60%), with minor formation of the D-valine epimer **8** (5%) (Figure 3, green traces). These results were in direct contrast with those obtained using PyAOP, where formation of the epimerised products **7** or **8** was favoured regardless of the *C*-terminal stereochemistry of the linear peptide precursor. While the stereoselective cyclisation of peptides has been reported to proceed independently of starting material configuration,[16] to the best of our knowledge, this is the first report of this phenomenon being reagent-controlled. The conformations of the different T3P and 1-hydroxy-7-azabenzotriazole (HOAt) active ester intermediates are speculated to mediate this process.

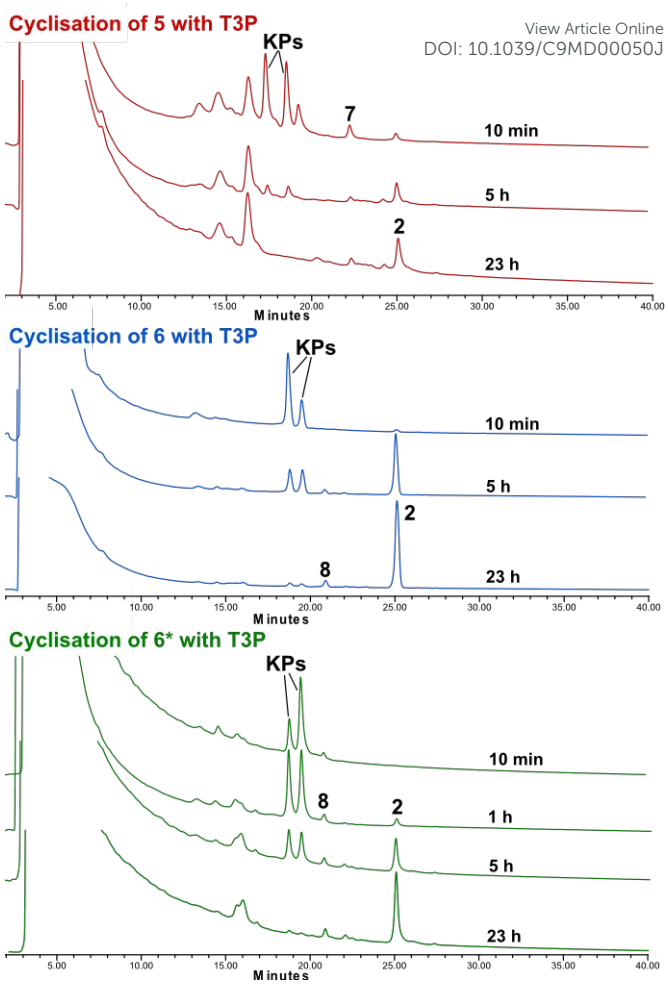
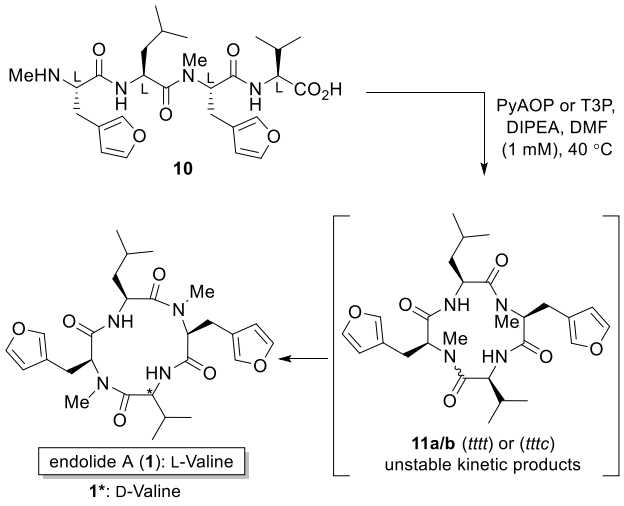


Figure 3 RP-HPLC (214 nm) profiles for the T3P mediated cyclisation of **5** (red, top), **6** (blue, middle) and **6*** (green, bottom) at 10 min, 1 h (for **6***), 5 h and 23 h. Linear gradient of 20–80% B over 40 min (~1% B min^{-1}) using a Luna C18 column (4.6 × 250 mm, 5 µm). A = 0.1% TFA in H_2O and B = 0.1% TFA in MeCN. KPs = kinetic products.

With endolide B (**2**) successfully in hand, we turned our attention to the preparation of endolide A (**1**). Since peptide **6** with a *C*-terminal valine residue gave the best results in the cyclisation to form endolide B (**2**), we chose to prepare linear peptide **10** with the sequence MeHN-3-(3-furyl)Ala-Leu-NMe-3-(3-furyl)Ala-Val-OH using the same procedures as outlined previously in Scheme 2. Peptide **10** was then cyclised using both T3P and PyAOP under the same conditions used in the

preparation of endolide B (**2**) [coupling reagent (3 equivalents) and DIPEA (6 equivalents) in DMF (1 mM) at 40 °C] (Scheme 4).



Scheme 4 Products observed during the cyclisation of linear tetrapeptide **10**.

After 23 hours, the reaction with T3P was observed to go to completion, affording a single major product (77%) with a retention time (27.5 min) much greater than that of the kinetic products **11a** and **11b** (20.0 and 21.6 min) (Figure 4, red traces). This product possessed a mass corresponding to that of the cyclised product (analysed by RP-HPLC and MS), and gratifyingly, MS and NMR analysis confirmed it to be endolide A (**1**) (following purification by preparative HPLC). The ^{1}H and ^{13}C NMR data were found to be in good agreement with the isolation report (see Tables S3 and S4 in the ESI).[1] In contrast, the reaction using PyAOP (Figure 4, blue traces) was observed to form a plethora of unidentifiable side products after 6 hours, together with trace formation of endolide A (**1**) (2%) and suspected formation of its D-valine epimer **1*** (9%), as a peak with the same retention time (22.8 min) was also observed in trace amounts (8%) in the previous cyclisation reaction using T3P.

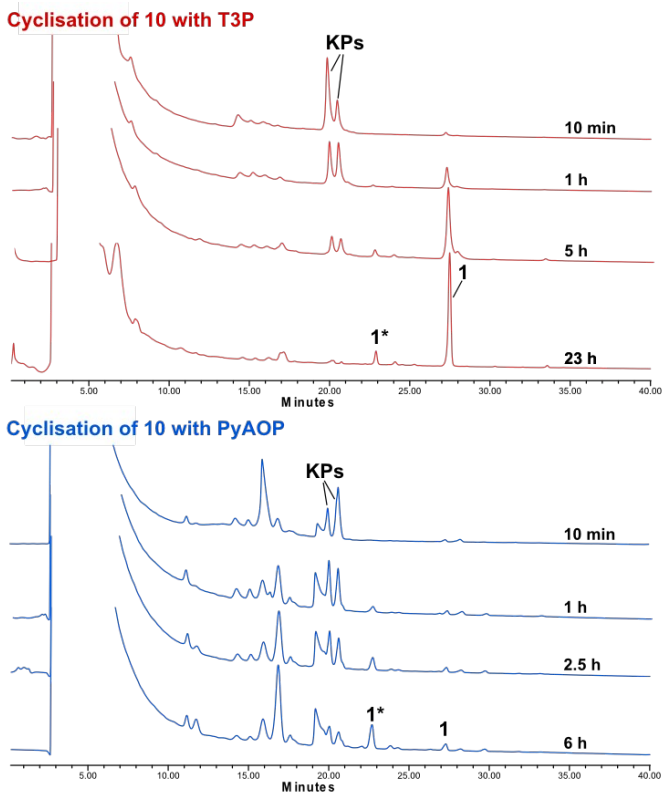


Figure 4 RP-HPLC (214 nm) profiles for the cyclisation of **10** with T3P (red, top) at 10 min, 1 h, 5 h and 23 h, and with PyAOP (blue, bottom) at 10 min, 1 h, 2.5 h and 6 h. Linear gradient of 20–80% B over 40 min (~1% B min^{-1}) using a Luna C18 column (4.6 × 250 mm, 5 µm). A = 0.1% TFA in H_2O and B = 0.1% TFA in MeCN. KPs = kinetic products.

Conclusions

The syntheses of endolides A (**1**) and B (**2**) were successfully achieved using a solution phase T3P-mediated cyclisation of the linear tetrapeptide precursors. The cyclisation reactions generated unstable cyclic kinetic products with inferred *tttt* and *tttc* conformations, which gradually isomerised to the more stable thermodynamic products possessing the native *tctc* conformations of the natural products.

While T3P was found to effect stereoselective cyclisations to provide endolide B (**2**) irrespective of the stereochemistry of the linear peptide precursors, PyAOP selectively formed the epimerised cyclic products. Further biological investigations of both endolides A and B (**1** and **2**) are underway.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are exceedingly grateful to Dr Celso Almeida (Endobios, Lisbon, Portugal) for the kind gift of the 3-(3-furyl)alanine building blocks used in this synthesis. We also wish to acknowledge the Maurice Wilkins Centre for Molecular Biodiscovery for financial support.

Notes and references

‡ L-3-(3-Furyl)alanine was obtained as a gift from Dr. Celso Almeida (Endobios, Lisbon, Portugal) and was Fmoc protected using standard procedures. See the ESI for further information.

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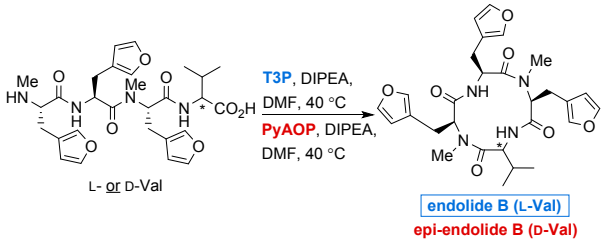

The first syntheses of the bioactive cyclic tetrapeptide natural products, endolides A and B, were accomplished using a solution-phase macrocyclisation reaction; the stereoselectivity of which was found to be reagent-controlled.