Peptido-organic Diels–Alder reactions on hydrophilic resin: scope for combinatorial chemistry

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Received (in Cambridge, UK) 24th April 2001, Accepted 5th October 2001
First published as an Advance Article on the web 14th November 2001

A simple route to activated dienes generated from enone precursors at the N-terminal of immobilised dipeptides is described. The dienes are subjected to reaction with a maleimide dieneophile containing a protected amino functionality for continued peptide synthesis. The reactions are pure and quantitative with dienes generated at the N-terminal of a variety of different dipeptides, including substrates containing amino acids with protected sidechain functionalities. In the work presented, hydrophilic POEPOP resin is used as a solid support. Also described in this paper is a high-yielding method for functional-group exchange of resin hydroxylic functionalities into azido groups *via* a Mitsunobu reaction, followed by reduction to yield amino-functionalised POEPOP.

Introduction

Peptides are natural substrates or ligands to numerous classes of the regulatory biomolecules. As a consequence of their regulatory function, such biomolecules are often involved in disease-related processes, and are interesting pharmaceutical targets. Generally, natural peptides are not useful as drug candidates due to their poor pharmakokinetic properties. However, the stability of peptides can be enhanced by different types of modifications. Thus, the introduction of structural constraints or amide-bond replacements have yielded compounds with improved pharmacokinetic properties.[1,2] In the field of protease inhibition, these efforts have resulted in the identification of potent compounds. These structures often resemble extended peptide structures displaying substituents or functionalities mimicking those of naturally occurring amino acids.[3] Also, many of these inhibitors contain various organic fragments in the 'backbone', such as electrophilic moieties or transition-state analogues.[4–7] Homo- or heterocyclo-hexanone moieties inserted into a peptide chain have previously been demonstrated to function as inhibitors of serine and cysteine proteases.[8,9] Also, bicyclic structures in peptide chains have been used as secondary-structure-inducing elements to target proteases,[10,11] and potent thrombin inhibitors of this type have been identified. Affinity between a protease and its substrates or inhibitors is obtained through numerous interactions along the entire binding cleft of the protease. Specificity results from these interactions.[2,3,12] In the 'one-bead-two-compounds' protease inhibitor assay, libraries of peptide isosteres are formed in solid-phase organic reactions and used in combination with immobilised fluorescence-quenched peptide substrates for on-bead screening for endoprotease inhibitors.[13–16] To maximise the interactions with the active cleft of the target endoprotease, the strategy is to form the organic fragment in a manner which allows the peptide synthesis to be continued after introduction. Based on these considerations, a solid-phase strategy was designed to incorporate bicyclic moieties into peptides *via* solid-phase Diels–Alder reactions, in a manner to comply with the library concept.

Since its discovery in 1928, the Diels–Alder reaction has been extensively utilised to generate a large variety of homo- or heterocyclic ring systems in inter- or intramolecular reactions.[17–20] Over the years, various heteroatom-substituted dienes were developed in an effort to design more reactive and regioselective dienes.[21] Also, since the advent of combinatorial chemistry, several examples of solid-phase Diels–Alder reactions using activated as well as non-activated dienes have appeared in the literature,[22–32] including examples using substrates of peptidic nature.[10,33,34]

Several novel polar resins were previously developed to provide solid supports which are chemically inert, and compatible with a wide range of solvents including aqueous conditions.[35,36] Thus, POEPOP, an all-ether-bonded PEG-based resin, was introduced to meet these demands and was employed throughout this study.

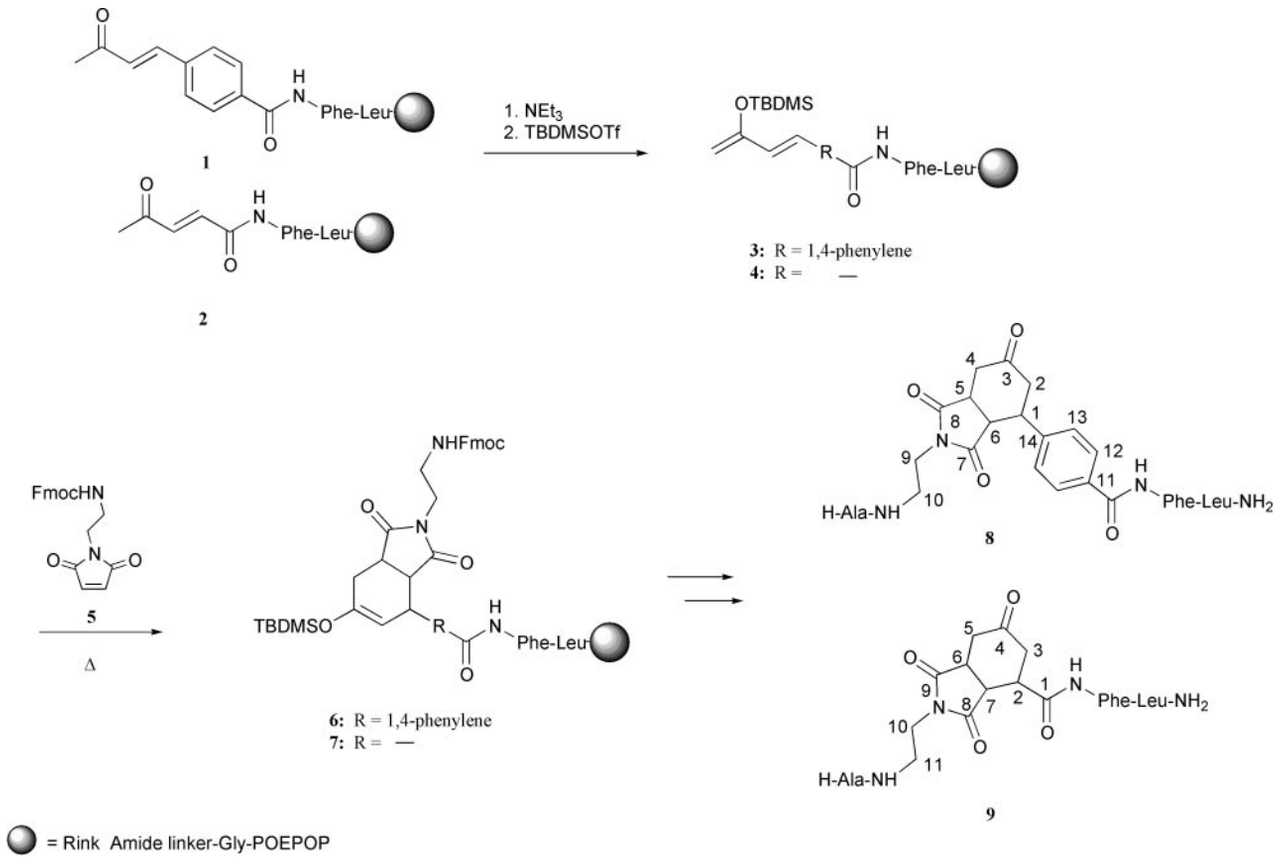
This paper describes the simple preparation of activated dienes from enone precursors at the N-termini of various immobilised dipeptides, and the reactions of these dienes with a maleimide dienophile to place bicyclic moieties at internal positions in a peptide. Furthermore, the conversion of resin hydroxy functionalities into amino functionalities for linker attachment *via* amide bonds is described, enabling compatibility with various reaction conditions, to be achieved.

Results and discussion

Resin functional-group exchange

Chemically inert $POEPOP_{1500}$ resin,[36] a PEG-based resin compatible with both aqueous and organic solvents, was utilised, in order to enable the use of a diverse range of chemical reaction conditions. The high chemical stability of POEPOP resin is immediately limited by the inate hydroxy functionalities of the resin, as these functionalities only allow easy attachment of linkers *via* labile ester bonds. To solve this, a procedure for attaching the hydroxymethylphenol (HMP) linker *via* an ether bond under Mitsunobu conditions[37] was previously described.[14] Analogously, the hydroxy functionalities of the POEPOP resin were transformed into azido groups using $(PhO)_2PON_3$ as the azide source.[38] After reduction of the azide with dithiothreitol,[39] amino functionalised $POEPOP_{1500}$ resin was obtained. The reduction was easily monitored by FT-IR, and the disappearance of the azide peak at 2115 cm^{-1} ensured full conversion. The final yield of the functional-group exchange was evaluated by coupling Fmoc-glycine to the amino groups followed by spectrophotometric quantification

 DOI: 10.1039/b103765j



Scheme 1 Preparation and Diels–Alder reactions of 1-peptidyl-3-silyloxybuta-1,3-dienes **3** and **4** with dienophile **5**. Numberings refer to numbering used in interpretation of NMR data.

of released piperidine–dibenzofulvene adduct from a known amount of resin upon Fmoc-deprotection. Generally, the yield was in the range of 75–100%, the higher yields being obtained when the solvent volume was kept at a minimum as previously described.[14] This functional-group exchange enabled attachment of the Rink amide linker *via* a chemically stable amide bond, hence allowing the use of a greater variety of reaction conditions.

Diels–Alder reactions

Diels–Alder reactions of immobilised dienes as well as immobilised dienophiles have previously been described.[10,11,22,24–34] For this investigation, a simple route from immobilised enones to activated, silyloxy substituted dienes reminiscent of Danishefsky's diene was envisaged, as opposed to previously described procedures for the preparation of non-activated, immobilised dienes.[34,40] Thus, to investigate the Diels–Alder reaction conditions, immobilised peptidyl enones **1** and **2** (Scheme 1) were synthesised and used as model substrates. The silyloxy dienes **3** and **4** were prepared by treating **1** and **2** sequentially with triethylamine and *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf) at 0 °C, followed by washings with dichloromethane (DCM). After drying, the resins was submitted to reaction with an electron-deficient dienophile. The quantitative conversions of these dienes in later reactions as observed by HPLC indicate that silyl-enolisation of the resin bound enones was complete. Several groups have reported Diels–Alder reactions of maleimides with immobilised non-activated or activated dienes, and generally the bicyclic products have been obtained in moderate to high yields.[27–32] To gain ready access to an amine handle for further peptide synthesis, *N*-[2-(fluoren-9-ylmethoxycarbonylamino)ethyl]-maleimide **5** was prepared[41] and used as a dienophile in reactions with **3** or **4** at 110 °C in toluene. The products **6** or **7** were obtained in quantitative conversions and high purities

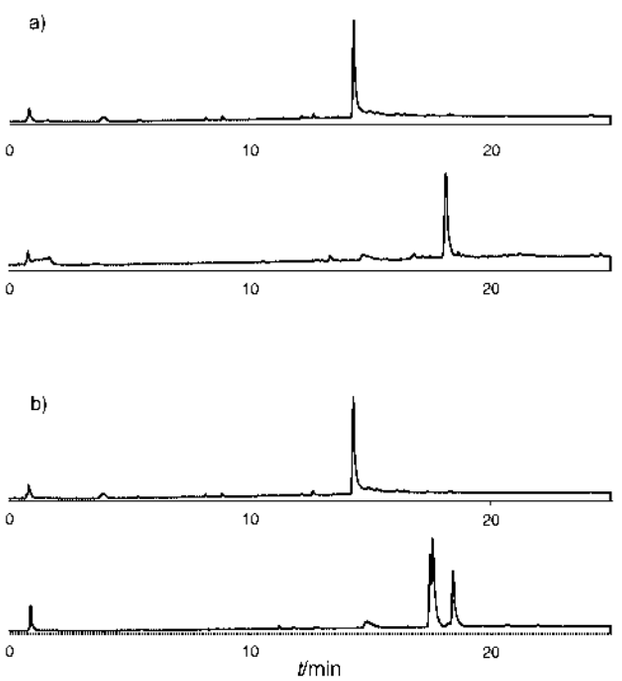
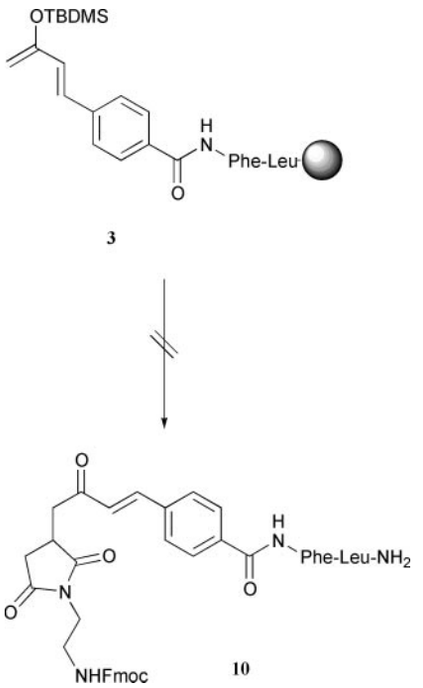


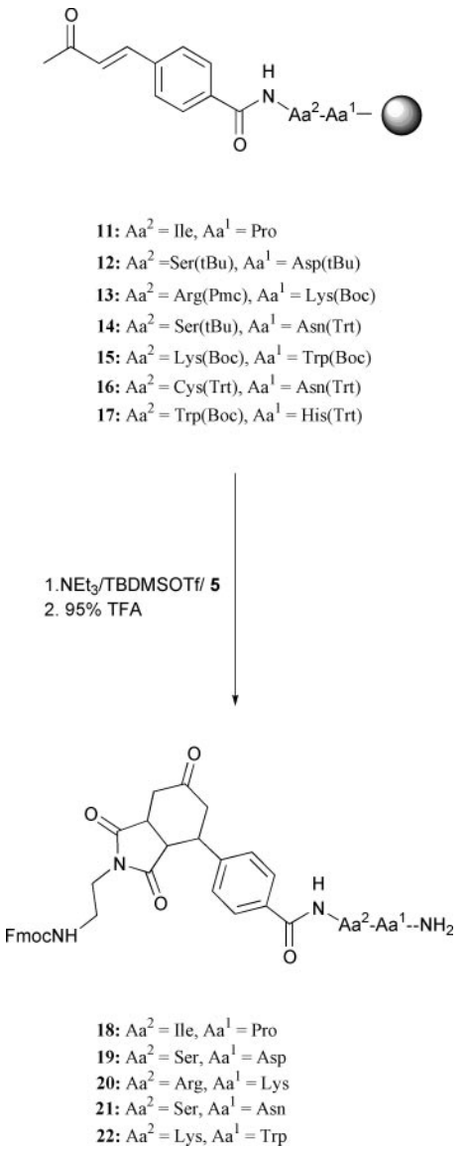
Fig. 1 (a) Chromatogram of **1** (upper) and crude Diels–Alder product **6** (lower). (b) Chromatogram of **2** (upper) and crude Diels–Alder product **7** (lower).

(Fig. 1). The non-cyclised, Michael-type adduct **10**, reported by other researchers,[32] was not observed in this case (Scheme 2). Products **6** and **7** were deprotected using the standard conditions of 20% piperidine in DMF, and the released amines were further acylated with Fmoc-Ala-OPfp ester. Following standard Fmoc deprotection, the products were cleaved from the solid phase to yield **8** and **9**, and analysed by NMR after



Scheme 2 Michael addition product **10** was not observed in the reaction between **3** and **5**.

HPLC purification. Solid-phase Diels–Alder reactions between activated or non-activated dienes and maleimides have been reported to proceed with *endo*-selectivity,[28,30,31] and in cases when dienes with chiral substituents were used, facial selectivity has been obtained. However, a completely non-stereospecific solid-phase Diels–Alder reaction between an activated, immobilised diene and substituted maleimides has also been reported.[32] In the case of **6**, the final product **8** obtained after acidic cleavage eluted as a single peak in the HPLC (Fig. 1), however, NMR investigation of **8** revealed the presence of two stereoisomers. Moreover, the final product obtained from **7** separated into 3 peaks on HPLC (Fig. 1), all having the mass of the expected product, and the presence of 3 stereoisomers was verified by NMR of products **9**. Hence, a certain stereocontrol was exerted in the reactions of **3** and **4** with **5**, as the possible number of diastereomers is 4 in each case. A series of structures similar to those of the bicyclic system **9** have previously been prepared in solid-phase Diels–Alder reactions and demonstrated to act as β-strand mimetics, and several potent inhibitors of thrombin have been identified from libraries of these compounds.[10,11] To investigate the scope of performing Diels–Alder reactions with a peptidic substrate, certain compatibility considerations were required. The chemistry performed had to be compatible with the presence of the protected functionalities of amino acid sidechains, and consequently, with the protecting groups normally used. Conversely, the peptides and the protecting groups also had to be stable to the reaction conditions. To investigate the Diels–Alder reaction of **5** further, a series of substrates **11**–**17** containing different amino acids were prepared and allowed to react with **5** under standard conditions after silyl enolisation (Scheme 3). Except for the reaction of **11**, which was chosen to represent a sterically congested substrate, the products from the reactions of **11**–**17** were less pure than those with **3**. However, when the reaction temperature was lowered from 110 °C to 90 °C, clean reactions were obtained with substrates **11**–**15** as illustrated in Fig. 2. Substrates **16** and **17** failed to perform well in the reactions (not shown in Fig. 2). This was ascribed to the presence of cysteine and histidine in these substrates, respectively, since Asn(Trt) and Trp(Boc) were tolerated in substrates **14** or **15**. Since the protecting groups used in substrates **11**–**17** were acid labile, their possible loss during the Diels–Alder reaction would not be detected in the



Scheme 3 Model reactions performed for elucidation of compatibility between Diels–Alder reactions of **5** and peptidyl dienes.

products upon acidic cleavage. However, the eventual loss of protecting groups would not be important unless it affected the further peptide synthesis. Hence, to investigate this the Diels–Alder products obtained with **11**–**17** were acylated prior to cleavage. It was evident that the Boc protecting group was unstable to the reaction conditions, as double acylations were observed in subsequent reactions of **13** and **15**. Bisacylations could be easily eliminated by re-introducing Boc using Boc_2O, subsequent to the Diels–Alder reactions and prior to Fmoc-deprotection and acylation. Taking these precausions, Diels–Alder reactions and further acylation of the products proceeded in high yields and purities, and the reaction is generally compatible with peptidic substrates.

Conclusions

In the work presented here, the simple solid-phase preparation and reactions of activated dienes from a simple enone precursor at the N-terminal of several immobilised dipeptides is described. The dienes were submitted to Diels–Alder reactions with a maleimide dienophile bearing a protected amine functionality suitable for a continued peptide synthesis. The reactions proceeded quantitatively to yield isomeric mixtures of Diels–Alder products. The compatibility of these reactions with the presence of amino acids bearing various protected sidechain functionalities was also investigated. Generally,

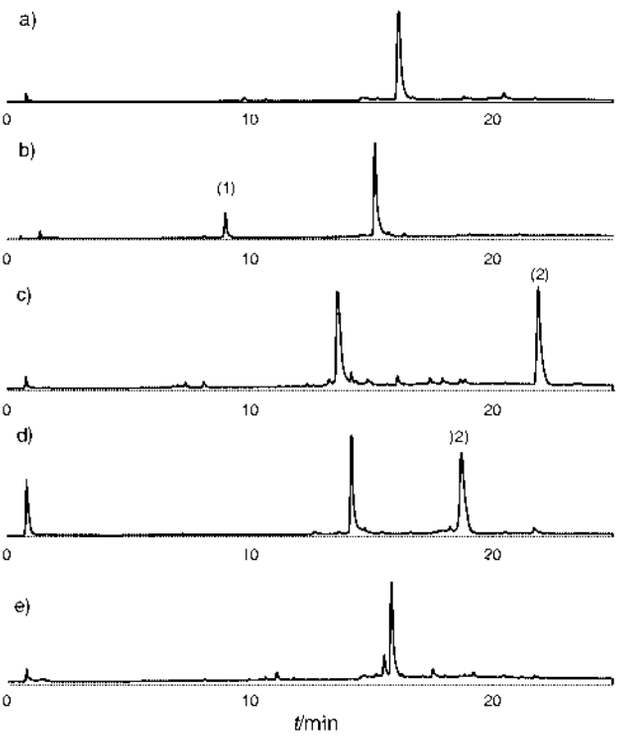


Fig. 2 (a)–(e) Chromatograms of crude products **18**–**22** from Diels–Alder reactions of silylenolised enones **11**–**15** and **5**. (b) Peak labelled (1) is starting material. (c) and (d) Peaks labelled (2) are cleaved protecting groups: (c) Pmc, (d) Trt.

quantitative and pure reactions were obtained with these substrates, except with a few amino acids or protecting groups which were not compatible with the Diels–Alder reaction conditions. This high degree of compatibility of the Diels–Alder reaction with peptidic substrates promoted the expansion to a combinatorial library format, as has recently been reported.[42] A method to transform the hydroxy functionalities of hydrophilic POEPOP resin used in the study into amino functionalities is described. This high-yielding transformation was accomplished in a Mitsunobu reaction using diphenylphosphoryl azide as the nucleophile followed by reduction. The functional group conversion greatly enhances the synthetic usefulness of POEPOP resin, as a wide variety of linkers can be attached *via* stable bonds, allowing the use of diverse chemical conditions to be used with the resin.

Experimental

All solvents were HPLC grade and were purchased from LabScan (Dublin, Ireland). DCM was dried by distillation from $CaCl_2$ and stored over 4 Å molecular sieves under inert gas. POEPOP resin was prepared according to literature procedures. Amino acids or their *N*-(fluoren-9-ylmethoxycarbonyl) (Fmoc)-amino acid pentafluorophenyl (Pfp) ester derivatives, *N*-(fluoren-9-ylmethoxycarbonyl)ethylendiamine hydrochloride and *N*-(fluoren-9-ylmethoxycarbonyl)succinimide were purchased from Bachem (Bubendorf, Switzerland) or Novabiochem (Läufelfingen, Switzerland). *N*-Ethylmorpholine (NEM), *N,N*-diisopropylethylamine (DIPEA), 4-formylbenzoic acid, *N*-(methoxycarbonyl)maleimide, TBDMSOTf, triphenylphosphine, diethyl azodicarboxylate (DEAD) and diphenylphosphoryl azide were purchased from Fluka (Buchs, Switzerland). Dithiothreitol and triethylamine were obtained from Aldrich (Helsinki, Finland). 3-Acetylacrylic acid was from Lancaster (Morecambe, England). Trifluoroacetic acid and benzylamine was purchased from Merch-Schuchardt (Hohenbrun, Germany) and piperidine was purchased from Riedel-de-Häen (Seelze, Germany). All reagents and solvents were used as received without further purification. Analytical RP-HPLC was performed on a Waters system (490E detector, two 510 pumps with gradient controller and id 8 mm RCM C_{18} column). Semi-preparative RP-HPLC was carried out on a Waters system (991 photodiode array detector and 600 E system controller) fitted with a Waters id 25 mm RCM C_{18} column. All RP-HPLC procedures were carried out using a linear gradient. Buffers were A: 0.1% TFA in water, B: 0.1% TFA in CH_3CN–water (9 : 1). Electrospray mass spectra were aquired on a Fisons VG Quattro 5098 spectrometer (mobile phase 50% aq. CH_3CN.) NMR spectra were recorded on a Bruker AMX 250 MHz spectrometer, a Varian Unity Inova 500 MHz spectrometer or a Bruker DRX 600 MHz spectrometer, and chemical shifts were referenced to residual solvent signals. All *J*-values are given in Hz.

Deprotection of N^{α}-Fmoc peptides was performed using 20% piperidine in DMF for 4 and 16 min. Washing volumes were always 1–2-times the volume neccesary to swell the resin. Minimum reaction volumes were used in all coupling reactions. Amino-group loadings were measured by determining the amount of released piperidine–dibenzofulvene adduct from a known sample spectrophotometrically at 190 nm using a standard curve. Amino-acid couplings were followed using the Kaiser test.[43]

4-(3-Oxobuten-1-yl)benzoic acid was prepared according to a literature procedure.[44] First crop of crystals yielded 37%.

N-[2-(Fluoren-9-ylmethoxycarbonylamino)ethyl]maleimide 5

N-(Fluoren-9-ylmethoxycarbonyl)ethylenediamine hydrochloride (1260 mg, 4 mmol) was suspended in saturated aq. $NaHCO_3$ (20 cm^3)–1,4-dioxane (20 cm^3) and the suspension was cooled to 0 °C. *N*-(Methoxycarbonyl)maleimide (775 mg, 5 mmol) was added and the mixture was stirred for 30 min at 0 °C, then at ambient temperature overnight. The solvent was evaporated off and the aqueous phase was extracted with EtOAc (3 × 20 cm^3). The organics were combined, dried over $MgSO_4$, and evaporated. Purification by flash chromatography on silica gel using 40% EtOAc in *n*-heptane as eluent. Yield was 800 mg (60%), ^{1}H NMR (250 MHz; $CDCl_3$) δ 3.36 (2H, t, *J* 6.0, Fmoc-NHCH_2), 3.64 (2H, t, *J* 6.0, CH_2 maleimide), 4.12 [1H, t, *J* 6.9, C*H*-(Fmoc)], 4.27 [2H, d, *J* 6.9, CH_2(Fmoc)], 6.63 (2H, s, vinyl), 7.25 [2H, m, Ar(Fmoc)], 7.33 [2H, m, Ar(Fmoc)], 7.51 [2H, m, Ar(Fmoc)], 7.69 [2H, m, Ar(Fmoc)]; ^{13}C NMR (62.5 MHz; $CDCl_3$) δ 38.0 (CH_2 maleimide), 40.3 (Fmoc-NH-*C*H_2), 47.4 (CH, Fmoc), 67.3 (CH_2, Fmoc), 120.2 (Ar, Fmoc), 125.4 (Ar, Fmoc), 127.3 (Ar, Fmoc), 128.0 (Ar, Fmoc), 134.5 (vinyl), 141.6 (Ar, Fmoc), 144.2 (Ar, Fmoc), 156.8 (CO, Fmoc), 171.1 (CO, maleimide); ESI-MS: Calc.: (*M*) = 362.4. Found: *m/z* (%) = 363.1 (15) (M + H^+), 385.0 (100) (M + Na^+).

o-[(*R,S*)-α-(Fluoren-9-ylmethoxycarbonylamino)-2′,4′-dimethoxybenzyl]phenoxyacetyl-('Rink amide')-glycyl-POEPOP$_{1500}$

POEPOP$_{1500}$ resin (2 g, loading 0.4 mmol g^{-1}) was washed successively with 1 M NaOH (3 × 40 cm^3), then with water (10 × 40 cm^3), acetone (5 × 40 cm^3) and DCM (3 × 40 cm^3) and dried under high vacuum. The dry resin was placed in a Schlenk-tube in an argon atmosphere and partly swollen in THF–DCM (1 : 1; 10 cm^3) and cooled to 0 °C. A solution of triphenylphosphine (2.4 g, 9 mmol) in THF–DCM (1 : 1; 6 cm^3) was added dropwise to a cold (0 °C) solution of DEAD (1.4 cm^3, 9 mmol) in THF–DCM (1 : 1; 9 cm^3), and the resulting brightly orange solution was transferred to the resin. Diphenylphosphoryl azide (2 cm^3, 9 mmol) was added to the reaction mixture and the reaction was run at 0 °C for 4 hours, and left overnight at room temperature. The resin was drained, washed with DCM (8 × 40 cm^3), and dried *in vacuo*. FT-IR: 2115 cm^{-1} (azide stretch). The resin was washed with DMF (3 × 40 cm^3) and suspended in a solution of dithiothreitol (3.08 g, 20 mmol) and DIPEA

(1.7 cm^3, 10 mmol) in DMF (10 cm^3), and agitated at 50 °C overnight. The resin was drained, and washed successively with DMF (5 × 40 cm^3) and DCM (5 × 40 cm^3). FT-IR of the resin showed no absorption at 2115 cm^{-1}. The resin was next coupled to Fmoc-Gly-OPfp ester (2.08 g, 4.5 mmol) in DMF (10 cm^3) and the reaction was followed using the Kaiser test. When the Kaiser test was negative the resin was washed successively with DMF (5 × 40 cm^3) and DCM (5 × 40 cm^3). The loading of amino functionalities was measured by cleavage of Fmoc from a known amount of resin using 20% piperidine in DMF followed by spectrophotometrical quantitation of the released fluorenylpiperidyl adduct at 290 nm and was shown to be 0.3 mmol g^{-1} (75%). The entire resin batch was deprotected using 20% piperidine in DMF followed by washes with DMF (5 × 40 cm^3). Rink amide linker (1460 mg, 2.7 mmol) and NEM (568 mm^3, 4.5 mmol) was dissolved in DMF (10 mm^3) and TBTU (809 mg, 2.52 mmol) was added. The mixture was kept at room temperature for 5 minutes prior to addition to the glycyl-$POEPOP_{1500}$ resin. The reaction was followed by the Kaiser test, and when negative the resin was drained, and washed successively with DMF (5 × 40 cm^3), water (5 × 40 cm^3), DMF (5 × 40 cm^3) and DCM (5 × 40 cm^3).

Resins **3** and **4** were prepared using the general procedure described for resin **3**.

4-[3-(*tert*-Butyldimethylsilyloxy)buta-1,3-dienyl]benzoyl-L-phenylalaninyl-L-leucyl-*p*-[(*R,S*)-α-methoxycarbonylamino-2,4-dimethoxybenzyl]phenoxyacetyl-glycyl-$POEPOP_{1500}$ 3

Fmoc-Rink amide-glycyl-$POEPOP_{1500}$ (500 mg, loading 0.3 mmol g^{-1}) was deprotected, and washed with DMF (5 × 10 cm^3). Fmoc-Leu-OPfp ester (389 mg, 0.75 mmol) was dissolved in DMF (2 cm^3) and added to the resin. The reaction was run at ambient temperature and was followed using the Kaiser test. When negative the resin was drained, and washed with DMF (5 × 10 cm^3), then deprotected, and washed with DMF. Fmoc-Phe-OPfp ester (415 mg, 0.75 mmol) was dissolved in DMF (2 cm^3) and added to the resin; when a negative Kaiser test was obtained the resin was drained, washed, deprotected and washed. 4-(3-Oxobut-1-enyl)benzoic acid (85 mg, 0.45 mmol) and NEM (95 mm^3, 0.75 mmol) were dissolved in DMF (2 cm^3) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (160 mg, 0.42 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt) (20 mg, 0.15 mmol) were added. The reaction mixture was allowed to preactivate for 5 minutes at ambient temperature prior to addition to the resin. When a negative Kaiser test was obtained, the resin was drained, washed successively with DMF (5 × 10 cm^3) and DCM (5 × 10 cm^3), and dried. The resin was placed in a flame-dried flask in an argon atmosphere and swollen in dry DCM (5.5 cm^3). After cooling of the resin to 0 °C, triethylamine (625 mm^3, 4.5 mmol) and *tert*-butyldimethylsilyl trifluoromethanesulfonate (775 mm^3, 3.38 mmol) were added sequentially. The mixture was gently stirred at 0 °C for 1 h and at room temperature for 1.5 h. The resin was drained and washed with DCM (6 × 10 cm^3) and dried *in vacuo*.

Diels–Alder reactions with *N*-[2-(9-fluoren-9-ylmethoxycarbonylamino)ethyl]maleimide **5** were performed as described for the preparation of **8**.

Diels–Alder reaction of 3 with *N*-[2-(fluoren-9-ylmethoxycarbonylamino)ethyl]maleimide 5

5 (135 mg, 0.375 mmol) was weighed into a screw-cap vial (Reacti Vial) and toluene (800 mm^3) was added. The vial was sealed and heated until compound **5** had dissolved, and **3** (50 mg, loading 0.3 mmol g^{-1}) was added and allowed to swell in the reagent solution. The vial was sealed and kept at 110 °C overnight, the resin was drained and washed successively with toluene (3 × 5 cm^{-3}) and DCM (5 × 5 cm^3). The product was deprotected using 20% piperidine–DMF and washed with DMF (6 × 5 cm^3). Fmoc-Ala-OPfp (36 mg, 0.075 mmol) was dissolved in DMF (500 mm^3) and added to the resin. The reaction was followed with the Kaiser test, and when complete the reagent was removed with suction and the resin was washed with DMF (6 × 5 cm^3) followed by deprotection, and washing with DMF as described above. Finally the resin was washed with DCM (6 × 5 cm^3) and the products were released using 10% TFA–DCM. The crude was purified on semipreparative HPLC to yield the products eluting as a single peak (2.1 mg total, 31%). NMR revealed two isomers; stereochemistry was not assigned.

8 Major isomer. ^{1}H NMR (500 MHz; CD_3CN–D_2O; 25 °C) δ 0.87 [3H, d, *J* 6.4, Me(Leu)], 0.90 [3H, d, *J* 6.4, Me(Leu)], 1.34 [3H, d, *J* 6.3, H-β(Ala)], 1.54 [1H, m, H-β(Leu)], 1.58 [1H, m, H-β(Leu)], 1.61 [1H, m, H-γ(Leu)], 2.56 (1H, m, H2), 2.61 (1H, m, H2), 2.77 (1H, m, H4), 2.99 (1H, m, H4), 3.07 [1H, m, H-β(Phe)], 3.20 (2H, m, H10), 3.27 [1H, m, H-β(Phe)] 3.46 (2H, m, H9), 3.52 (1H, m, H5), 3.56 (1H, m, H6), 3.82 [1H, q, *J* 6.3, H-α(Ala)], 3.90 (1H, m, H1), 4.29 [1H, m, H-α(Leu)], 4.77 [1H, m, H-α(Phe)], 7.22 [1H, m, H-π(Phe)], 7.27 [2H, m, H-ε(Phe)], 7.32 [2H, m, H-δ(Phe)], 7.32 (2H, m, H13), 7.69 (2H, m, H12); ^{13}C NMR (125 MHz; CD_3CN–D_2O; 25 °C) δ 16.9 [C-β(Ala)], 21.5 [C-δ(Leu)], 23.2 [C-δ(Leu)], 25.3 [C-γ(Leu)], 37.6 [C-β-(Phe)], 37.8 (C10), 38.1 (C4), 38.5 (C1), 38.8 (C5), 38.9 (C9), 41.0 [C-β(Leu)], 44.5 (C6), 49.8 [C-α(Ala)], 52.4 [C-α(Leu)], 56.0 [C-α(Phe)], 127.3 [C-π(Phe)], 127.7 (C12), 128.7 (C13), 129.0 [C-ε(Phe)], 130.0 [C-δ(Phe)], 133.0 (C11), 138.3 [C-γ(Phe)], 144.5 (C14), 168.2 (*C*OAr), 170.1 [CO(Ala)], 172.3 [CO(Phe)], 175.5 [CO(Leu)], 179.6 (C8), 179.7 (C7), 210.1 (C3).

8 Minor isomer. ^{1}H NMR (500 MHz; CD_3CN–D_2O; 25 °C) δ 0.87 [3H, d, *J* 6.4, Me(Leu)], 0.90 [3H, d, *J* 6.4, Me(Leu)], 1.38 [3H, d, *J* 6.3, H-β(Ala)], 1.54 [1H, m, H-β(Leu)], 1.58 [1H, m, H-β(Leu)], 1.61 [1H, m, H-γ(Leu)], 2.56 (1H, m, H2), 2.65 (1H, m, H2), 2.77 (1H, m, H4), 2.99 (1H, m, H4), 3.07 [1H, m, H-β(Phe)], 3.20 (2H, m, H10), 3.27 [1H, m, H-β(Phe)], 3.46 (2H, m, H9), 3.52 (1H, m, H5), 3.56 (1H, m, H6), 3.80 [1H, q, *J* 6.3, H-α(Ala)], 3.90 (1H, m, H1), 4.29 [1H, m, H-α(Leu)], 4.77 [1H, m, H-α(Phe)], 7.22 [1H, m, H-π(Phe)], 7.27 [2H, m, H-ε(Phe)], 7.32 [2H, m, H-δ(Phe)], 7.39 (2H, m, H13), 7.71 (2H, m, H12); ^{13}C NMR (125 MHz; CD_3CN–D_2O, 25 °C) δ 16.8 [C-β(Ala)], 21.5 [C-δ(Leu)], 23.2 [C-δ(Leu)], 25.3 [C-γ(Leu)], 37.6 [C-β-(Phe)], 37.8 (C10), 38.1 (C4), 38.5 (C1), 38.8 (C5), 38.9 (C9), 41.0 [C-β(Leu)], 44.5 (C6), 49.8 [C-α(Ala)], 52.4 [C-α(Leu)], 56.0 [C-α(Phe)], 127.3 [C-π(Phe)], 128.3 (C12), 128.4 (C13), 129.0 [C-ε(Phe)], 130.0 [C-δ(Phe)], 133.2 (C11), 138.3 [C-γ(Phe)], 146.7 (C14), 168.2 (*C*OAr), 170.2 [CO(Ala)], 172.3 [CO(Phe)], 175.5 [CO(Leu)], 179.6 (C8), 179.7 (C7), 210.4 (C3); ESI-MS: Calc.: (*M*) = 660.3. Found: *m*/*z* (%) = 661.3 (25) ($M + H^+$), 683.3 (100) ($M + Na^+$).

Compound **9** was obtained as 3 isomeric products separating on HPLC (stereochemistry not assigned):

9 Major isomer. Yield: 2.6 mg (19%); ^{1}H NMR (500 MHz; CD_3CN–D_2O; 25 °C) δ 0.85 [3H, d, *J* 6.1, Me(Leu)], 0.89 [3H, d, *J* 6.1, Me(Leu)], 1.32 [3H, d, *J* 6.4, H-β(Ala)], 1.56 [1H, m, H-γ(Leu)], 1.67 [2H, m, H-β(Leu)], 2.12 (1H, m, H3), 2.23 (1H, m, H3), 2.43 (1H, m, H5), 2.60 (1H, m, H5), 2.90 [1H, m, H-β(Phe)], 2.97 (1H, m, H2), 3.20 [1H, m, H-β(Phe)], 3.23 (1H, m, H11), 3.31 (1H, m, H6), 3.34 (1H, m, H7), 3.51 (1H, m, H10), 3.52 (1H, m, H11), 3.55 (1H, m, H10), 3.81 [1H, q, *J* 6.4, H-α(Ala)], 4.25 [1H, m, H-α(Leu)], 4.58 [1H, m, H-α(Phe)], 7.21 [1H, m, H-π(Phe)], 7.21 [2H, m, H-δ(Phe)], 7.27 [2H, m, H-ε(Phe)]; ^{13}C NMR (125 MHz; CD_3CN–D_2O; 25 °C) δ 15.1 [C-β(Ala)], 19.3 [C-δ(Leu)], 21.4 [C-δ(Leu)], 23.0 [C-γ(Leu)], 35.2 [C-β(Phe)], 35.3 (C6), 35.6 (C11), 35.7 (C5), 37.7 (C10), 38.3 (C2), 38.4 [C-β(Leu)], 38.7 (C3), 39.8 (C7), 47.7 [C-α(Ala)], 50.6 (C-α(Leu)], 53.9 [C-α(Phe)], 125.6 [C-π(Phe)], 127.2 [C-ε(Phe)], 128.0 [C-δ(Phe)], 136.0 [C-γ(Phe)], 169.0 [CO(Ala)],

171.0 [CO(Phe)], 172.0 (C1), 174.8 [CO(Leu)], 177.7 (C9), 177.9 (C8), 206.8 (C4); ESI-MS: Calc.(M) = 584.7. Found: m/z (%) = 586.3 (100) ($M + H^+$), 608.3 (15) ($M + Na^+$).

9 Minor isomer a. Yield: 1.9 mg (14%). ^{1}H NMR (500 MHz; CD_3CN–D_2O; 25 °C) δ 0.85 [3H, d, J 6.4, Me(Leu)], 0.88 [3H, d, J 6.4, Me(Leu)], 1.34 [3H, d, J 6.3, H-β(Ala)], 1.52 [1H, m, H-β(Leu)], 1.57 [1H, m, H-β(Leu)], 1.61 [1H, m, H-γ(Leu)], 2.23 (1H, m, H5), 2.41 (1H, m, H3), 2.45 (1H, m, H5), 2.57 (1H, m, H3), 2.81 [1H, m, H-β(Phe)], 3.16 [1H, m, H-β(Phe)], 3.27 (1H, m, H11), 3.33 (1H, m, H2), 3.36 1H, m, H11), 3.38 (1H, m, H7), 3.45 (1H, m, H6), 3.52 (2H, m, H10), 3.81 [1H, q, J 6.3, H-α(Ala)], 4.23 [1H, m, H-α(Leu)], 4.52 [1H, m, H-α(Phe)], 7.13 [2H, m, H-δ(Phe)], 7.20 [1H, m, H-π(Phe)], 7.22 [2H, m, H-ε(Phe)]. ^{13}C NMR (125 MHz; CD_3CN–D_2O; 25 °C) δ 17.1 [C-β(Ala)], 21.6 [C-δ(Leu)], 23.3 [C-δ(Leu)], 25.4 [C-γ(Leu)], 37.6 C11), 37.7 C2), 37.8 C3), 38.0 [C-β(Phe)], 39.1 C10), 40.1 C6), 40.2 C5), 40.4 C7), 41.0 [C-β(Leu)], 49.9 [C-α(Ala)], 52.8 [C-α(Leu)], 55.6 [C-α(Phe)], 127.7 [C-π(Phe)], 129.5 [C-ε(Phe)], 130.0 [C-δ(Phe)], 137.9 [C-γ(Phe)], 170.7 [CO(Ala)], 172.5 [CO(Phe)], 174.1 (C1), 174.3 (C9), 180.2 (C8), 208.8 (C4); ESI-MS: Calc.: (M) = 584.7. Found: m/z (%) = 585.79 (100) ($M + H^+$).

9 Minor isomer b. Yield: 1.5 mg (11%); ^{1}H NMR (500 MHz; CD_3CN–D_2O; 25 °C) δ 0.79 [3H, d, J 6.4, Me(Leu)], 0.84 [3H, d, J 6.1, Me(Leu)], 1.34 [3H, d, J 6.3, H-β(Ala)], 1.45 [1H, m, H-γ(Leu)], 1.49 [2H, m, H-β(Leu)], 2.46 (1H, m, H5), 2.54 (1H, m, H5), 2.66 (1H, m, H2), 2.94 [1H, m, H-β(Phe)], 3.02 [1H, m, H-β(Phe)], 3.32 (2H, m, H11), 3.38 (1H, m, H7), 3.40 (1H, m, H6), 3.49 (2H, m, H10), 3.79 [1H, q, J 6.3, H-α(Ala)], 4.17 [1H, m, H-α(Leu)], 4.37 [1H, m, H-α(Phe)], 7.21 [2H, m, H-δ(Phe)], 7.23 [1H, m, H-π(Phe)], 7.27 [2H, m, H-ε(Phe)]; ^{13}C NMR (125 MHz; CD_3CN–D_2O; 25 °C) δ 15.0 [C-β(Ala)], 19.3 [C-δ(Leu)], 21.2 [C-δ(Leu)], 23.0 [C-γ(Leu)], 35.4 [C-β(Phe)], 35.5 (C7), 35.6 (C2), 35.7 (C11), 37.1 (C10), 38.4 (C6), 38.5 (C5), 38.9 [C-β(Leu)], 47.7 [C-α(Ala)], 50.3 [C-α(Leu)], 54.1 [C-α(Phe)], 125.6 [C-π(Phe)], 127.3 [C-ε(Phe)], 127.7 [C-δ(Phe)], 135.4 [C-γ(phe)], 168.7 [CO(Ala)], 170.4 [CO(Phe)], 177.1 (C9), 178.5 (C8); ESI-MS: Calc.: (M) = 584.7. Found: m/z (%) = 585.79 (100) ($M + H^+$).

18: ESI-MS: Calc.: (M) = 761.9. Found: m/z (%) = 762.4 (100) ($M + H^+$), 784.2 (48) ($M + Na^+$).

19: ESI-MS: Calc.: (M) = 753.8. Found: m/z (%) = 754.3 (50) ($M + H^+$).

20: ESI-MS: Calc.: (M) = 836.0. Found: m/z (%) = 418.9 (100) ($M + 2H^+$), 836.4 (17) ($M + H^+$).

21: ESI-MS: Calc.: (M) = 752.8. Found: m/z (%) = 753.5 (20) ($M + H^+$).

22: ESI-MS: Calc.: (M) = 866.0. Found: m/z (%) = 866.3 (90) ($M + H^+$).

Acknowledgements

We thank Bent O. Petersen, Dr Charlotte H. Gotfredsen and Dr Axel Meissner, Carlsberg Laboratory, for recording high-field NMR data. Dr Morten Grøtli, Carlsberg Laboratory is thanked for fruitful discussions. The Danish National Research Foundation is gratefully acknowledged for financial support.

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