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Stereoselective synthesis of a novel pseudopeptide hapten for the generation of hydrolytic catalytic antibodies

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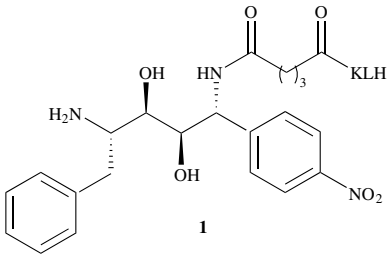
Abstract—The synthesis of a novel hapten containing a 1,4-diamino-2,3-diolbutane core unit is described. The molecule contains four stereogenic centres and has been synthesised via a stereoselective route. The absolute stereochemistry of each stereogenic carbon atom has been assigned by nuclear Overhauser effect experiments carried out on a number of derivatives.

1. Introduction

The production of artificial receptors that can achieve recognition at the molecular level is an important goal of organic and bioorganic chemistry. The study of catalytic antibodies is certainly at the forefront of the search for a wide range of novel enzyme-like mimics, and aims to assemble together the basic concept of enzyme catalysis with the possibility of the immune system of generating antibodies against virtually any molecule. This research field has undergone a rapid development process in the last decade.[1,2] From the initial 'proof of concept' and demonstration of fundamental enzyme-like characteristics, antibodies have been shown to catalyse a remarkably broad range of organic transformations, including difficult and unfavourable chemical reactions and the ones that are not catalysed by enzymes. Moreover research efforts have recently focused on the in-depth study of the structural and mechanistic basis of antibody catalysis and on the combinatorial processes involved in the immune response itself. This has allowed investigators to begin to understand the important interactions taking place within the active site and to evaluate the relationship existing between immunogen structure and the structure of the catalytic site formed. As a result, more emphasis has been put on improving hapten design, which seems to influence the structure and efficiency of the antibody generated. These studies have highlighted the potential of polyclonal antibodies as a valuable tool for the evaluation of hapten design, since their use allows assessment of the entire immune response.

Our previous work[3,4] used anionic phosphate and phosphonate haptens to produce sheep polyclonal catalytic antibody preparations with hydrolytic activity towards carbonate, ester and amide substrates. These preparations effect the catalysis of the reactions of these substrates with hydroxide ion assisted by hydrogen bond donation at the reaction centre by high pK_a side-chains (tyrosine and arginine).[5] In an attempt to produce antibodies with an active centre carboxyl group to supply acid/base or nucleophilic catalysis in acidic media, we are investigating the use of cationic haptens.



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Herein we report the stereoselective synthesis of the cationic immunogen **1** and the assignment of the absolute configuration of the four stereogenic centres.

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2. Results and discussion

2.1. Design and synthesis of immunogen 1

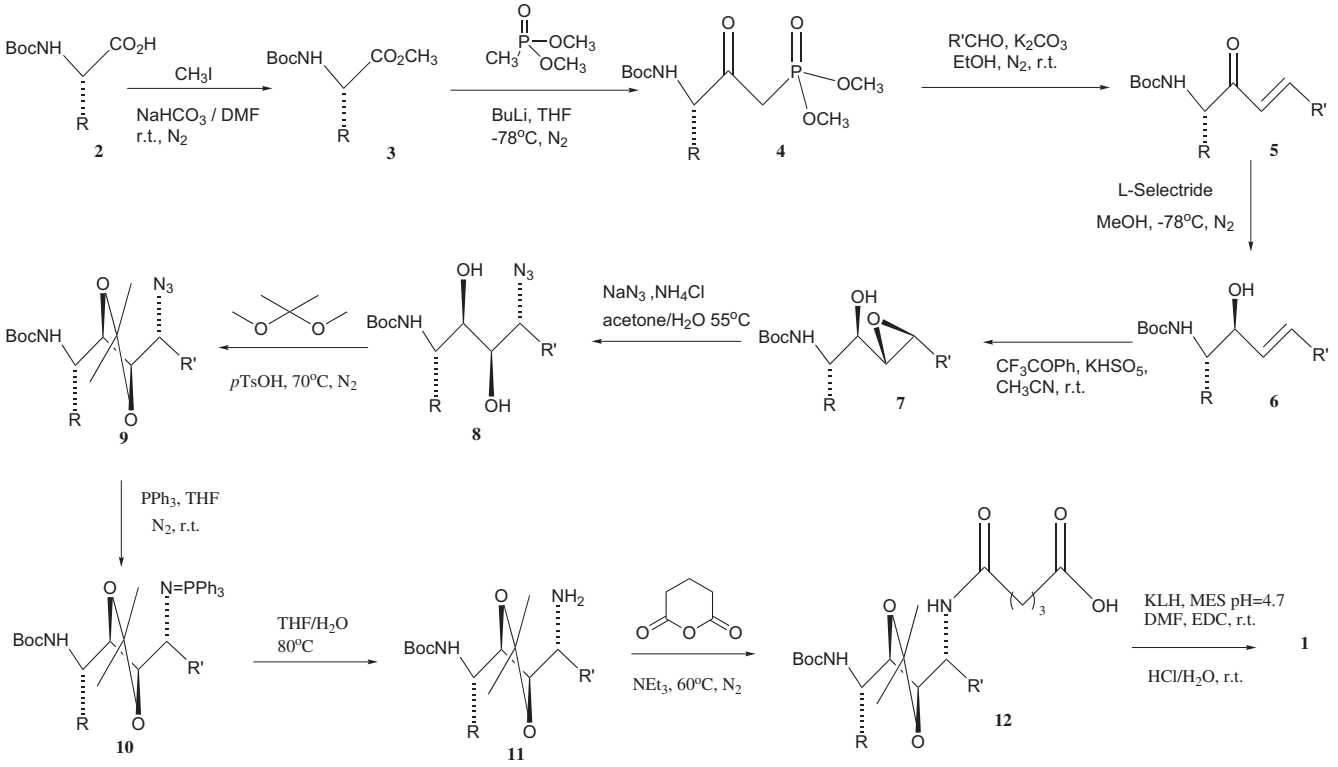
The design of immunogen **1**, incorporating the 1,4-diamino-2,3-diolbutane core unit, was inspired by studies on mechanism-based inhibitors for the HIV-1 protease, an enzyme belonging to the family of the aspartic proteases and characterised by two catalytically essential aspartate residues in the active site.[6–8] A number of molecules containing this functional moiety have been shown to be effective inhibitors showing IC_{50} values between 0.2 and 0.4 nM.[9,10]

Significant structural features of the hapten included: (i) the two amino groups, expected to be protonated in vivo and able to recruit a basic residue, such as an aspartate, in the antibody site[6–8]; (ii) the secondary hydroxyl groups, included to mimic the tetrahedral transition state, while attempting to recruit other hydrogen bonding residues;[11] (iii) the 4-nitrophenyl moiety, a highly antigenic residue in the immunogen and also a chromogenic leaving group[12] (as 4-nitrophenolate) to be introduced in the substrates; (iv) the presence of the two aromatic rings, contributing to the formation of hydrophobic pockets in the active site of the antibody, that would favour the substrate–antibody binding. Furthermore the hapten contains a C_2 pseudosymmetry axis that may elicit a symmetrical active site in the antibody resembling the active site of the HIV protease.[13] The nonleaving aromatic moiety, a benzyl group, allows us to mimic phenylalanine, resulting in the hapten being a pseudopeptide. Since the hapten would be used for the generation of polyclonal antibodies, we chose to synthesise the immunogen as a single stereoisomer to minimise the heterogeneity of the antibody preparations.

The synthetic route, shown in Scheme 1, was broadly inspired by work carried out on similar molecules containing the same 1,4-diamino-2,3-diol core unit.[14] The presence of the 4-nitrophenyl moiety in the structure of hapten **1**, with its significant electron-inductive effect, altered the expected reactivity and prompted a number of changes in the original route, in order to obtain the target molecule **1**, containing four stereogenic centres, as a single stereoisomer.

The first step of the synthesis was the methylation of *N*-Boc-L-Phe with methyl iodide and $NaHCO_3$ to give the corresponding ester **3**. This reacted with the carbanion generated by reaction of dimethylmethyl phosphonate with butyl lithium to form the β-ketophosphonate **4**. Comparison of the experimentally determined value of the specific rotation for **4** with the literature data confirmed that there was no evidence of racemisation for this substrate.[15] Phosphonate **4** underwent a Horner–Wadsworth–Emmons reaction, reacting with *p*-nitrobenzaldehyde and K_2CO_3 in ethanol to give the α,β-unsaturated ketone **5**. The *trans* isomer was exclusively obtained, as shown by the coupling constant of the alkene protons, $J = 16.2$ Hz.[16]

The reduction of the ketone in **5** represented a crucial step in the stereoselective synthesis because it would create the second stereogenic centre in the molecule,



Scheme 1. Synthetic route to hapten **1**, where R = benzyl and R′ = 4-nitrophenyl.

expected to further control the stereochemistry on the formation of the other two remaining stereogenic centres. A large number of reducing reagents in different conditions were tried, among which L-Selectride provided the most significant results in terms of diastereoselectivity, summarised in Table 1. The ratio of the two diastereoisomers was determined by ^{1}H NMR, from the integration values of the proton attached to the carbon that bears the hydroxylic group for each diastereoisomer.

Table 1. Experimental conditions investigated for the stereoselective reduction of the ketone moiety in **5**

Hydride	Solvent	Temperature (°C)	Ratio (*S*,*R*)/(*S*,*S*)
$NaBH_4$	MeOH	0	1:1
DIBAL	THF	−78	No reaction
L-Selectride	THF	−78	7:3
L-Selectride	CH_2Cl_2	−78	4:6
L-Selectride	THF	0	6:4
L-Selectride	CH_2Cl_2	0	5:5
L-Selectride	MeOH	−78	8.5:1.5

Diastereoselective reductions of *N*-protected α-aminoketones have been often reported in the literature, but the selectivity is hardly predictable in a general way.[17] A number of factors are believed to contribute to the stereoselectivity: the presence of chelating metals, the bulkiness of the reducing agent and the nature of the solvent, which is particularly important in the L-Selectride reductions.[18] When the reduction of α-amino enones is carried out by chelating reagents such as aluminium hydrides, or in the presence of chelating metals, *anti*-reduction is often observed, resulting from the hydride attack on the less hindered side of the chelate conformation of the substrate, as shown in Figure 1. On the contrary, in the absence of chelating metals, or if the amino group is doubly protected by bulky alkyl groups, the reaction most often occurs via the standard Felkin–Ahn conformation b, thus leading to *syn* products.[17,19] Nevertheless, *anti*-reductions occurring via the cyclic Cram conformation c have also been observed.[14]

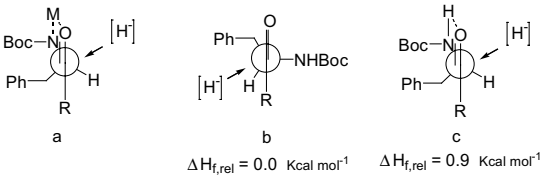


Figure 1. The three possible conformations of the enone **5**.

Since L-Selectride is not a chelating reagent, model a can be ruled out in our case, and the *syn* selectivity observed when the reaction is carried out at −78 °C in THF, or even better in methanol, can be explained using the Felkin–Ahn model b. A lack of selectivity is observed when dichloromethane is used as solvent under the same conditions. We have performed a conformational analysis on enone **5**. The calculations were carried out with the AM1 Hamiltonian[20] and the systematic search yielded 13 minimum energy conformations. The absolute minimum corresponds to the expected Felkin–Ahn conformation b, and is immediately followed by a cyclic Cram conformation of type c, which is higher in energy by only 0.9 Kcal/mol. This conformation is likely to be even more disfavoured in hydrogen bond acceptor solvents such as THF, or donor solvents such as methanol, which destroy the intramolecular hydrogen bond. On the contrary, on moving to a solvent such as dichloromethane, which is not hydrogen bonding, conformations b and c are expected to be very close in energy, thus offering an explanation for the lack of selectivity in such solvents.

Although a small amount of the main diastereoisomer of **6** was obtained pure by chromatography and used for characterisation, in the standard procedure the mixture of the two diastereoisomers was used for the following step, the epoxidation of **6** to give **7**. An initial attempt using *m*-CPBA[21] did not provide the desired product probably due to the electron-deficient characteristics of alkene **6**. Use of H_2O_2/NaOH led to a mixture with by-products.[22] Epoxide **7** was successfully obtained following reaction with CF_3COPh and oxone®. Oxone® is a triple salt composed of $2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$ and its active ingredient is potassium peroxymonosulfate, $KHSO_5$.[23] The in situ generated dioxirane reacted with the double bond in **6**, to give epoxide **7** as a single diastereoisomer in 60% yield after purification. The isolation of a single product indicates that the minor diastereoisomer of **6** present in the reaction mixture is lost during the purification procedure.

Reaction of **7** with NaN_3/NH_4Cl to open the epoxide ring provided azide **8** in good yields. The nucleophilic reaction is regiospecific on the benzylic carbon and also highly stereoselective since the NMR of the isolated product shows the presence of a single diastereoisomer.

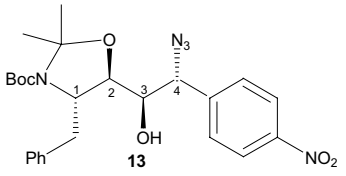
At this stage protection of the diol in **8** was required in order to avoid formation of aziridine derivatives when reducing the azide group to amine,[24] using PPh_3 in THF followed by hydrolysis. In the next step **11** was reacted with glutaric anhydride to introduce the linker between the hapten and the carrier protein. The coupling of **12** to the carrier protein KLH was successfully carried out with EDC in 0.1 M MES at pH = 4.7 and DMF, following standard experimental procedures.[25] Finally, deprotection of the Boc and isopropylidene ketal groups was obtained by suspending the conjugate in HCl (0.2 M) for 24 h. These experimental conditions were previously tested using the BSA conjugate of hapten **12** and by carrying out ELISA experiments with polyclonal antibodies readily available in our laboratory. The resulting data confirmed that both the coupling and deprotection had taken place successfully. Immunogen **1** is currently being used in a standard immunisation programme for the generation of sheep polyclonal antibodies.

2.2. Determination of the absolute configuration of the stereogenic centres of the hapten by NMR

In this section the stereogenic carbon atoms under investigation have been numbered as shown in the structure below for easier understanding.

The first stereogenic centre, C^1, has a known configuration, since the starting material for the synthesis is enantiomerically pure and it has been demonstrated that no racemisation occurs during the course of the synthetic pathway. Knowing the absolute configuration for C^1 allowed us to assign the absolute configuration for the other stereogenic centres in the molecule by performing a series of nuclear Overhauser effect (NOE) experiments on different intermediates of the synthesis.

For the determination of C^2, **13** was used because of the restricted rotation between the C^1–C^2 bond.



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The experiments carried out on **13** were based on the measurement of the magnitude of the NOE between the proton attached to C^1 (called H^1) and the proton attached to C^2 (called H^2).

The existence of an NOE between the two protons is shown by a cross peak in the two-dimensional NOESY NMR experiment. The magnitude of the Overhauser enhancement between the two protons (here H^1 and H^2) can be estimated from the initial slope of a plot of cross peak intensity (its volume) versus mixing time τ_m. The mixing time is a variable time in the NOESY pulse sequence, during which the two hydrogens cross relax (exchange magnetisation). This initial slope (M_{12}) depends upon the cross relaxation rate, which in turn is proportional to the inverse sixth power of the internuclear distance (r_{12}).[26,27] This is an established methodology that has been reviewed.[28,29]

In order to be able to quantify the H^1–H^2 distance it is necessary to construct a similar plot for a pair of protons with known separation that is a reference distance. This is provided by a pair of *ortho* protons on the aromatic ring, separated by 2.46 Å. The experiments were performed at low temperature, since the hindered amide-type rotation about the amide bond in the Boc group resulted in broad lines at ambient temperature and so interfered with the measurement of the NOE effect. At low temperature, 223 K, this rotation was sufficiently slowed to result in well-resolved ^{1}H NMR spectra. The spectra were measured with mixing times 50, 100, 150, 200 and 250 ms. The cross peak volumes between the *ortho* protons (Ar) from either side of the diagonal were averaged at each mixing time, as were the cross peak volumes between H^1 and H^2. The two plots of volume versus mixing time were linear and fitted to straight lines, and gave slopes $Ar = 0.0322 \pm 0.0058$, H^1–$H^2 = 0.0122 \pm 0.0019$. This gave the ratio of the *distances* Ar/H^1–$H^2 = 0.85 \pm 0.05$.

The 3D molecular modelling program Chem 3D was used to calculate the theoretical atomic distances between the *ortho* aromatic protons in **13**, and between H^1–H^2 for both possible configurations at C^2 (*R*, H^1–H^2 *trans*; *S*, H^1–H^2 *cis*). These calculated distances were Ar = 2.455 Å, H^1–H^2 *trans* = 2.90 Å, H^1–H^2 *cis* = 2.37 Å. Therefore the calculated ratio of the distances Ar/H^1–H^2 *trans* = 0.85 is in excellent agreement with the experimental data, whereas the calculated ratio Ar/H^1–H^2 *cis* = 1.04 is outside the limits of the experimental data.

Therefore, sufficient evidence has been obtained to state that H^1 and H^2 present a *trans* disposition in **13**.

This indicates that reduction of the ketone **5** has led to the *syn* product **6**. The diastereoselectivity obtained is in accordance with the Felkin–Ahn model and is supported by literature data.[30]

In order to determine the stereochemistry of C^3, the protected diol **9** was used because it presented restricted rotation about the C^2–C^3 bond.

A set of experiments similar to the ones carried out on **13** for the determination of the configuration on C^2 was performed on **9**. The intensities of the NOE cross peaks were measured at mixing times 200, 400, 600 and 800 ms. These longer mixing times than used for **13** above gave better signal/noise in the spectra, but the plots of the cross peak volumes versus mixing time extends into the nonlinear region and required the data be fitted to an exponential of the form:

$$V = V_\infty\{1 - \exp(-A\tau_m)\}$$

where V is the cross peak volume at mixing time τ_m, V_∞ and A are constants to be determined for each NOE build-up curve. The initial slope (limit $\tau_m \to 0$) is given by the product $A \cdot V_\infty$.

The reference distance was taken this time from the benzylic protons (Bz), and the distance to be determined is H^2–H^3. The initial slopes from the fitted data were $Bz = 0.0572 \pm 0.0045$, and H^2–$H^3 = 0.0170 \pm 0.0035$. This gave the ratio[31] of the *distances* Bz/H^2–$H^3 = 0.82 \pm 0.04$.

As before the program Chem 3D was used for the calculation of the distances H^2–H^3 in **9** for the configurations (*S*,*R*,*S*) and (*S*,*R*,*R*) corresponding to the carbons (C^1,C^2,C^3). The configuration C^3 (*S*) corresponds to H^2–H^3 *trans*, and C^3 (*R*) to H^2–H^3 *cis*. The calculated distances were Bz = 1.79 Å, H^2–H^3 *trans* = 3.08 Å and H^2–H^3 *cis* = 2.33 Å. The calculated ratio Bz/ H^2–H^3 *trans* = 0.58 is very different to the experimental value, but the calculated ratio Bz/H^2–H^3 *cis* = 0.77 is very close to the experimental limit. Therefore, it can be concluded that the configuration for C^3 in **9** is *R*. This establishes that the epoxide is formed *syn* to the OH group, possibly favoured by coordination of the oxirane reagent to the OH substituent. The consequence of this is that the configuration of the four stereogenic centres in epoxide **7** results being (*S*,*R*,*R*,*S*), respectively, for (C^1,C^2,C^3, C^4).

Having determined the stereochemistry of the epoxide ring this leads to the assignment of the configuration of the last stereogenic centre in the final compound, formed by the opening of the epoxide ring with sodium azide. The attack of the nucleophile on C^4 will be on the side opposite to the oxygen bridge and will involve the inversion of the configuration of the carbon to which the nucleophile will be finally attached, therefore providing an (*R*)-configuration for C^4.

The remaining synthetic steps leading to the target hapten do not affect the stereochemistry of any of the centres. Furthermore the isolation of a single stereoisomer product with each subsequent reaction leading from **8** to **12** confirms that the stereochemistry is preserved. Any racemisation occurring at this stage would lead to formation of diastereoisomers, easily detectable in the reaction mixture.

3. Conclusions

The stereoselective synthesis of the diamino diol hapten **1** never reported before has been established, together with the absolute configuration of the stereogenic centres, found to be (C^1,C^2,C^3,C^4): (*S*,*R*,*R*,*R*). This hapten is currently used for the first time in an immunisation programme for the generation of sheep polyclonal catalytic antibodies.

4. Experimental

4.1. General remarks

All the chemicals used in synthesis were purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK), Lancaster Chemical Co. (Morecambe, Lancashire, UK), Sigma Chemical Co. (Poole, Dorset, UK) and Merck Ltd (Lutterworth, Leicestershire, UK). Melting points were measured on a Reichert microscope melting point apparatus (Reichert, Austria). Optical rotation was measured with a polarimeter from Optical activity Ltd (Huntingdon, UK) and all measurements were done at 25 °C Model AA-100. NMR experiments were performed on a JEOL EX270, on a Bruker AM250, on a Bruker AMX-400 and on a Bruker Avance-600. Mass spectra were recorded on a ZAB-SE4F FAB + mass spectrometer.

4.2. Methyl (2*S*)-3-phenyl-2-[*N*-(*tert*-butyloxycarbonyl)-amino]propanoate 3

Under a nitrogen atmosphere *N*-Boc-L-Phe (0.500 g, 1.78 mmol) was added to THF (4 mL); the solution was then stirred for 5 min at 25 °C. $NaHCO_3$ solid (0.470 g, 5.59 mmol) was added followed by the addition of CH_3I (0.350 mL, 5.62 mmol) dropwise. The reaction was stirred for 18 h at room temperature until the TLC monitoring system [AcOEt–PE (15:85)] showed no increase in the intensity of the product spot. The solvent was removed in vacuo. The residue was partitioned between AcOEt (30 mL) and water (10 mL). The aqueous phase was acidified to pH 3 with HCl 1 M and extracted with AcOEt (3 × 40 mL). The combined organic extracts were washed with a saturated solution of NaCl (40 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness. Ester **3** was obtained as a brown oil (0.480 g, 91% yield) [R_f 0.40 AcOEt–PE (15:85)]. Although a small sample was further purified for characterisation, the crude product was considered pure enough to be used in the next step without further purification.

$[\alpha]_D = -4.2$ (*c* 1.43, MeOH) [lit.[31] $[\alpha]_D = -4.6$ (*c* 1.0, MeOH)]; ^{1}H NMR ($CDCl_3$, 270 MHz): δ 7.0–7.3 (m, 5H), δ 4.9 (br, 1H), δ 4.5–4.6 (m, 1H), δ 3.6 (s, 3H), δ 2.8–3.1 (m, 2H), δ 1.3 (s, 9H); MS (FAB, *m*/*z*): 280 ($[MH]^+$), 243 and 180.

4.3. Dimethyl [(3*S*)-4-phenyl-3-[*N*-(*tert*-butyloxycarbonyl)amino]-2-oxobutyl]phosphonate 4

Butyl lithium solution in hexane (1.6 M, 13.4 mL, 21.5 mmol) was added dropwise to a stirred solution of dimethyl methanephosphonate (2.3 mL, 21 mmol) in THF (20 mL) under a nitrogen atmosphere at −78 °C. The solution was stirred for 10 min at −78 °C. A solution of ester **3** (1.000 g, 3.579 mmol) in THF (5 mL) was added dropwise and stirred for 2 h at −78 °C, whereupon TLC monitoring (AcOEt) showed that the reaction had gone to completion and the product formed had an $R_f = 0.41$. Water (10 mL) was added to the reaction solution and the aqueous layer was neutralised with HCl 1 M. The THF and the water were removed in vacuo and the residue was partitioned between AcOEt (30 mL) and water (10 mL). The aqueous layer was extracted with AcOEt (3 × 30 mL). The combined organic fractions were washed with a saturated solution of NaCl (10 mL), dried over $MgSO_4$ and concentrated to dryness. The desired product was afforded in 90% yield (1.200 g) as a brown oil pure enough to be directly used in the next step. A small sample was further purified for full characterisation.

Mp 74–76 °C [lit.[15] 74–76 °C]; $[\alpha]_D = -10.5$ (*c* 2.65, $CHCl_3$) [lit.[15] $[\alpha]_D = -5.94$ (*c* 1.80, $CHCl_3$)]; ^{1}H NMR ($CDCl_3$, 270 MHz): δ 7.1–7.3 (m, 5H), δ 5.4 (d, $J = 8.0$ Hz, 1H), δ 4.5–4.6 (m, 1H), δ 3.8 (d, $J = 11.3$ Hz, 3H), δ 3.8 (d, $J = 11.3$ Hz, 3H), δ 2.9–3.4 (m, 4H), δ 1.3 (s, 9H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 201.2, 155.3, 136.5, 129.4, 128.7, 127.0, 80.2, 61.3, 53.2, 37.6, 37.0, 28.3; MS (FAB, *m*/*z*): 372 ($[MH]^+$), 316 and 272.

4.4. (1*E*,4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-1-(4-nitrophenyl)-5-phenylpent-1-en-3-one 5

p-Nitrobenzaldehyde (0.352 g, 2.51 mmol) was added to a stirred solution of phosphonate **4** (1.150 g, 3.096 mmol) in EtOH (42 mL) under a nitrogen atmosphere at room temperature. K_2CO_3 (0.560 g, 4.05 mmol) was added in small portions over a period of 15 min. The reaction was stirred for 1 h whereupon TLC

monitoring (AcOEt) showed that phosphonate **4** was consumed and that there was still some aldehyde that had not reacted (eluting system AcOEt–PE 1:9 running the TLC plate twice). The reacting solution was filtered and neutralised with glacial acetic acid if necessary. The solvent was removed in vacuo. The residue was partitioned between AcOEt (45 mL) and saturated $Na_2S_2O_5$ (15 mL). The aqueous phase was extracted with AcOEt (3×45 mL). The combined organic layers were washed with saturated $Na_2S_2O_5$ (30 mL) until the TLC monitoring system [AcOEt–PE (1:9) running the TLC plate twice] showed that there was no *p*-nitrobenzaldehyde in the organic layer. The combined organic layers were washed with saturated $NaHCO_3$ (50 mL) and saturated NaCl (50 mL), dried over $MgSO_4$, filtered and concentrated giving **5** as a yellow solid after recrystallisation in di-isopropyl ether (0.560 g, 60% yield) [R_f 0.16; AcOEt–PE (1:9) running the TLC plate twice].

Mp 120–122 °C; $[\alpha]_D = -3.0$ (*c* 1.59, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.2 (d, $J = 9.0$ Hz, 2H), δ 7.6 (d, $J = 16.2$ Hz, 1H), δ 7.6 (d, $J = 9.0$ Hz, 2H), δ 7.1–7.4 (m, 5H), δ 6.7 (d, $J = 16.2$ Hz, 1H), δ 5.3 (d, $J = 7.1$ Hz, 1H), δ 4.8–5.0 (m, 1H), δ 3.1 (d, $J = 6.7$ Hz, 2H), δ 1.4 (s, 9H); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 197, 155, 148, 141, 140, 136, 130, 129, 128, 126, 125, 123, 81, 60, 38, 28; MS (FAB, *m*/*z*): 397 ($[MH]^+$), 341 and 297.

4.5. (1*E*,3*S*,4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-1-(4-nitrophenyl)-5-phenylpent-1-en-3-ol 6

Under a nitrogen atmosphere, enone **6** (100.0 mg, 0.2522 mmol) was dissolved in freshly distilled MeOH (40 mL) at −78 °C. L-Selectride solution (1 M, 0.760 mL, 0.760 mmol) was added and the resulting solution was left stirring for 1 h at −78 °C, whereupon TLC monitoring [AcOEt–PE (3:7)] showed that the reaction had gone to completion. The reaction was acidified to pH 5 with a 1 M HCl solution and the solvent was removed by rotary evaporation. The residue was partitioned between AcOEt (30 mL) and saturated $NaHCO_3$ solution (10 mL) and the aqueous layer was extracted with AcOEt (3×30 mL). The combined organic phases were washed with saturated NaCl solution (40 mL), dried over $MgSO_4$ and concentrated in vacuo. Purification of the residue by flash chromatography [eluting solvent AcOEt–PE (2:8)] gave the mixture of diastereoisomers (*S*,*S*) and (*S*,*R*) on a 8.5:1.5 ratio (78.4 mg, 78% yield). [R_f 0.35; AcOEt–PE (3:7)]. A small sample of the main diastereosisomer (*S*,*S*) was obtained pure from the chromatography and used to obtain the characterisation data described below. The minor diastereoisomer is present in very small quantities and has not been isolated pure.

Compound **6** (*S*,*S*): mp 110–112 °C; $[\alpha]_D = -15.9$ (*c* 2.08, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.1 (d, $J = 9.0$ Hz, 2H), δ 7.5 (d, $J = 9.0$ Hz, 2H), δ 7.1–7.3 (m, 5H), δ 6.7 (d, $J = 14.5$ Hz, 1H), δ 6.3 (dd, $J = 6.2$, 14.5 Hz, 1H), δ 4.9 (d, $J = 9.8$ Hz, 1H), δ 4.3 (br, 1H), δ 3.9 (br, 1H), δ 3.1 (br, 1H), δ 2.9 (d, $J = 6.7$ Hz, 2H), δ 1.3 (s, 9H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 156.3, 147.0, 143.3, 138.0, 135.1, 129.3, 128.7, 127.1, 126.7, 124.0, 79.9, 72.3, 60.5, 56.3, 38.0, 28.3; MS (FAB, *m*/*z*): 399 ($[MH]^+$), 343 and 325.

4.6. (1*S*,2*S*,3*R*,4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-1,2-epoxy-1-(4-nitrophenyl)-5-phenylpentan-3-ol 7

Trifluoroacetophenone (0.860 mL, 6.12 mmol) was added to a stirred solution of alcohol **7** (0.500 g, 1.25 mmol) (mixture of the two diastereoisomers) in acetonitrile (35 mL). A 4×10^{-4} M EDTA solution (23.2 mL, 9.28×10^{-3} mmol) was added as well as a solid mixture of $NaHCO_3$ (1.750 g, 20.83 mmol) and oxone (3.930 g, 6.392 mmol) in small portions over a 10 min period. The reaction was left stirring overnight excluding the flask from light. The TLC monitoring system [R_f 0.29; AcOEt–PE (15:85), running the TLC plate three times] showed that the reaction had gone to completion after 30 h. The solvent was evaporated and the resulting reaction crude was partitioned between AcOEt (30 mL) and water (10 mL). The aqueous phase was extracted with AcOEt (3×30 mL) and the combined organic extracts were washed with brine (30 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure to provide an oil that was further purified by flash chromatography. The purification [eluting system AcOEt–PE (2:8)] provided product **7**, as a single diastereoisomer, as a white solid in 60% yield (0.312 g).

Mp 119–120 °C; $[\alpha]_D = -22.8$ (*c* 0.94, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.2 (d, $J = 8.6$ Hz, 2H), δ 7.4 (d, $J = 8.6$ Hz, 2H), δ 7.1–7.3 (m, 5H), δ 4.9 (d, $J = 9.2$ Hz, 1H), $\delta = 4.1$ (br, 1H), δ 3.8–4.0 (m, 1H), δ 3.8–3.9 (m, 1H), δ 3.0 (br, 1H), δ 2.8–3.0 (m, 2H), δ 2.8 (d, $J = 3.2$ Hz, 1H), δ 1.3 (s, 9H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): 156, 148, 144, 138, 130, 129, 127, 126, 124, 81, 69, 64, 55, 54, 39, 28; MS (FAB, *m*/*z*): 415 ($[MH]^+$), 359 and 315.

4.7. (1*R*,2*R*,3*R*,4*S*)-1-Azido-4-[*N*-(*tert*-butyloxycarbonyl)amino]-1-(4-nitrophenyl)-5-phenylpentane-2,3-diol 8

Epoxide **7** (0.350 g, 0.844 mmol) was dissolved in a biphasic system composed of acetone (10.8 mL) and water (5.4 mL). A mixture of NaN_3 (0.740 g, 11.3 mmol) and NH_4Cl (0.611 g, 11.3 mmol) was added as a solid. The reacting solution was refluxed at 55 °C for 30 h. Diol **8** was detected by the TLC monitoring system [R_f 0.56; AcOEt–PE (3:7), running the TLC plate twice] and the solvent pair was rotary evaporated. The residue was partitioned between AcOEt (60 mL) and water (20 mL). The aqueous phase was extracted with AcOEt (3×60 mL) and the combined organic extracts were washed with NaCl saturated solution (40 mL), dried over $MgSO_4$ anhydrous, filtered and concentrated to dryness. The resultant oily product was purified by flash chromatography [eluting solvent AcOEt–PE (15:85)] to give **8** in 80% (0.310 g) as off-white solid.

Mp 130–132 °C; $[\alpha]_D = +40.4$ (*c* 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.2 (d, $J = 8.8$ Hz, 2H), δ 7.6 (d,

$J = 8.8$ Hz, 2H), δ 7.2–7.4 (m, 5H), δ 4.8 (d, $J = 5.6$ Hz, 1H), δ 4.7 (d, $J = 8.6$ Hz, 1H), δ 4.4 (d, $J = 4.2$ Hz, 1H), δ 3.9–4.1 (m, 1H), δ 3.5–3.7 (m, 1H), δ 3.2–3.4 (m, 1H), δ 3.0 (d, $J = 5.6$ Hz, 1H), δ 2.7–3.0 (m, 2H), δ 1.3 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 157.7, 147.9, 143.6, 137.4, 129.3, 128.9, 128.6, 126.7, 123.6, 80.9, 73.4, 73.1, 66.8, 52.3, 37.3, 28.1; MS (FAB, m/z): 458 ($[MH]^+$), 358, 164 and 119.

4.8. (*S*)-1-{(4*R*,5*R*)-5-[(*R*)-Azido-(4-nitrophenyl)methyl]-2,2-dimethyl-1,3-dioxolan-4-yl}-*N*-(*tert*-butyloxycarbonyl)-2-phenylethylamine 9 and (1*R*,2*R*)-2-azido-1-[(4*S*,5*R*)-3-(*tert*-butyloxycarbonyl)amino-2,2-dimethyl-1,3-oxazolan-5-yl]-2-(4-nitrophenyl)-ethanol 13

p-Toluenesulfonic acid (40.0 mg, 0.232 mmol) was added to a stirred solution of azide **8** (1.030 g, 2.251 mmol) in dimethoxypropane (11.2 mL, 91.5 mmol). The mixture was refluxed at 70 °C and after a period of 1 h the reaction had reached completion, as determined by TLC monitoring system [AcOEt–PE (2:8)], $R_F = 0.50$. The solvent was evaporated and the resultant residue was extracted with AcOEt (50 mL) and saturated $NaHCO_3$ aqueous solution (20 mL). The aqueous phase was extracted with AcOEt (3×50 mL) and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography [eluting system AcOEt–PE (5:95)] affording **9** as a white solid in 55% (0.610 g) yield and **13** as a white solid in 28% (0.310 g).

Compound **9**: mp 72–74 °C; $[\alpha]_D = +15.7$ (c 1.03, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.2 (d, $J = 8.6$ Hz, 2H), δ 7.4 (d, $J = 8.6$ Hz, 2H), δ 7.2–7.4 (m, 5H), δ 4.9 (d, $J = 7.8$ Hz, 1H), δ 4.9 (d, $J = 10.2$ Hz, 1H), δ 4.2–4.4 (m, 1H), δ 4.1 (d, $J = 7.0$ Hz, 1H), δ 4.0 (dd, $J = 6.7$, 10.2 Hz, 1H), δ 2.7–3.0 (m, 2H), δ 1.4 (s, 9H), δ 1.3 (s, 3H), δ 1.2 (s, 3H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 161.5, 151.4, 144.2, 136.0, 135.7, 135.4, 133.1, 132.1, 130.6, 115.2, 86.3, 69.7, 56.6, 47.7, 36.9, 35.0, 34.8, 33.2, 30.8; MS (FAB, m/z): 498 ($[MH]^+$), 398, 278 and 220.

Compound **13**: mp 68–71 °C; $[\alpha]_D = +64.5$ (c 1.14, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.2 (d, $J = 8.3$ Hz, 2H), δ 7.4 (d, $J = 8.4$ Hz, 2H), δ 7.1 (br, 3H), δ 6.9 (br, 2H), δ 4.7 (d, $J = 3.8$ Hz, 1H), δ 4.2 (br, 1H), δ 3.8–3.9 (m, 1H), δ 3.3–3.4 (m, 1H), δ 3.0 (br, 2H), δ 2.7 (br, 1H), δ 1.6 (s, 6H), δ 1.5 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 151.0, 150.0, 146.9, 141.2, 136.1, 128.8, 127.2, 125.6, 122.4, 93.7, 79.5, 73.1, 64.9, 59.8, 38.4, 35.9, 27.5, 25.7; MS (FAB, m/z): 498 ($[MH^+]$), 406 and 306.

4.9. (*S*)-1-{(4*R*,5*R*)-5-[(*R*)-Triphenyliminophosphorano-(4-nitrophenyl)methyl]-2,2-dimethyl-1,3-dioxolan-4-yl}-*N*-(*tert*-butyloxycarbonyl)-2-phenylethylamine 10

To a solution of azide **9** (0.210 g, 0.422 mmol) in freshly distilled THF (38 mL) under a nitrogen atmosphere, PPh_3 (0.120 g, 0.457 mmol) was added. The solution was left stirring over a period of 24 h. Upon disappearance of the starting material monitored by TLC (AcOEt, $R_f = 0.27$), water (52 μL, 2.8 mmol) was added and the solution was stirred at room temperature for 24 h. The reaction solution was concentrated to dryness and the residue was partitioned between AcOEt (60 mL) and water (20 mL). The aqueous layer was extracted with AcOEt (3×60 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the crude by flash chromatography [eluting system AcOEt–PE (5:5)] provided iminophosphorane **10** in 65% yield (0.200 g) as a foamy beige solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 7.6 (d, $J = 8.8$ Hz, 2H), δ 7.0–7.4 (m, 20H), δ 6.9 (d, $J = 8.7$ Hz, 2H), δ 5.0 (br, 1H), δ 4.5–4.6 (m, 1H), δ 4.4–4.5 (m, 2H), δ 4.1–4.3 (m, 1H), δ 2.9 (dd, $J = 5.9$, 13.2 Hz, 1H), δ 2.7 (dd, $J = 9.1$, 13.3 Hz, 1H), δ 1.3 (s, 9H), δ 1.1 (s, 6H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 154.9, 153.0, 146.0, 138.5, 132.4, 132.3, 131.1, 129.8, 128.4, 128.2, 126.0, 122.8, 107.8, 83.1, 78.5, 58.0, 51.1, 39.7, 28.6, 26.6, 24.6; ^{31}P NMR ($CDCl_3$, 109.3 MHz): δ 9.1; MS (FAB, m/z): 733 ($[MH]^+$), 278 and 262.

4.10. (*S*)-1-{(4*R*,5*R*)-5-[(*R*)-Amino-(4-nitrophenyl)methyl]-2,2-dimethyl-1,3-dioxolan-4-yl}-*N*-(*tert*-butyloxycarbonyl)-2-phenylethylamine 11

To a stirred solution of azide **9** (0.360 g, 0.723 mmol) in anhydrous THF (66 mL) under a nitrogen atmosphere, PPh_3 (0.210 g, 0.801 mmol) was added. The solution was left stirring over a period of 24 h. Upon disappearance of the starting material monitored by TLC {[AcOEt–PE (3:7)], $R_f = 0.05$}, water (68 mL) was added and the solution was refluxed at 80 °C for 24 h. After this period, the TLC monitoring system [AcOEt–PE (3:7) running the TLC plate twice] showed a spot at $R_f = 0.39$ corresponding to amine **11**. The solvent pair was evaporated and the residue was partitioned between AcOEt (60 mL) and water (20 mL). The aqueous layer was extracted with AcOEt (3×60 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the crude product by flash chromatography [eluting system AcOEt–PE (3:7)] provided amine **11** (0.205 g, 60% yield) as a foamy beige solid.

Mp 112–115 °C; $[\alpha]_D = -25.3$ (c 4.14, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.1 (d, $J = 8.6$ Hz, 2H), δ 7.4 (d, $J = 8.6$ Hz, 2H), δ 7.2–7.4 (m, 5H), δ 4.9 (d, $J = 9.8$ Hz, 1H), δ 4.4–4.6 (m, 1H), δ 4.0–4.3 (m, 3H), δ 2.8–3.0 (m, 2H), δ 1.5 (br, 2H), δ 1.4 (s, 9H), δ 1.2 (s, 6H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 155.4, 152.3, 147.3, 138.2, 129.7, 128.6, 127.9, 126.6, 124.0, 108.3, 80.4, 79.7, 54.2, 50.3, 41.3, 28.6, 26.9, 24.4; MS (FAB, m/z): 472 ($[MH]^+$), 416 and 372. Anal. Calcd for $C_{25}H_{33}O_6N_3$: C, 63.8; H, 7.0; N, 8.9 required; C, 59.9; H, 6.3; N, 6.8 found.

4.11. 5-{(*R*)[(4*R*,5*R*)-5-(1*S*)-1-[*N*-(*tert*-butyloxycarbonyl)-amino]-2-phenylethyl-2,2-dimethyl-1,3-dioxolan-4-yl]-(4-nitrophenyl)methylamino}-5-oxopentanoic acid 12

To a solution of amine **11** (100.0 mg, 0.2120 mmol) in freshly distilled THF (20 mL) under a nitrogen

atmosphere, glutaric anhydride (40.0 mg, 0.351 mmol) and NEt_3 (60 μL, 0.43 mmol) were added. The mixture was refluxed at 60 °C for a period of 22 h, after which the reaction had gone to completion, TLC monitoring system [AcOEt–PE (6:4)], $R_f = 0.24$. After evaporation of the solvent, the residue was partitioned between AcOEt (50 mL) and water at pH = 5 (15 mL). The aqueous phase was extracted with AcOEt (3 × 50 mL) keeping the pH of the aqueous phase at 5. The combined organic extracts were dried over $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by flash chromatography using a gradient AcOEt–cyclohexane (2:8) to AcOEt–cyclohexane (5:5) to afford a dark green oil (77.0 mg, 62% yield).

^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.1 (d, $J = 8.5$ Hz, 2H), δ 7.4 (d, $J = 8.5$ Hz, 2H), δ 7.0–7.3 (m, 5H), δ 4.7–5.0 (m, 3H), δ 4.0–4.2 (m, 3H), δ 2.6–2.9 (m, 2H), δ 2.3 (t, $J = 6.8$ Hz, 2H), δ 2.2 (t, $J = 7.2$ Hz, 2H), δ 1.7–1.9 (m, 2H), δ 1.4 (s, 9H), δ 1.3 (s, 6H); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 176.8, 172.8, 156.3, 147.7, 147.3, 137.8, 129.6, 128.8, 128.3, 127.0, 123.8, 108.7, 80.9, 76.6, 54.4, 51.0, 41.1, 35.1, 33.3, 28.6, 27.2, 24.7, 20.5; MS (FAB, m/z): 608 ([MNa]$^+$), 586 ([MH]$^+$), 486 and 472. Anal. Calcd for $C_{30}H_{39}O_9N_3$: C, 61.5; H, 6.7; N, 7.2 required; C, 60.9; H, 6.8; N, 8.4 found.

4.12. Coupling of 12 to KLH

To KLH (25.0 mg, 3.7×10^{-6} mmol) reconstituted in water (2.5 mL), 0.1 M PBS pH = 7.3 (5 mL) was added. After the solution was stirred gently, acid **12** (20.0 mg, 3.4×10^{-2} mmol) previously dissolved in DMF (1.5 mL), was added dropwise followed by the addition of 0.1 M MES pH = 4.7 (5 mL). EDC (25.0 mg, 0.13 mmol) was added portionwise and the mixture was left stirring for a period of 5 h. Upon completion of the reaction, the reacting solution was dialysed against water using dialysis tubing-Visking size 8 32/32 in. and freeze dried.

4.13. Deprotection of the amino and isopropylidene ketal groups in conjugate of 12 to KLH

The conjugate KLH-**12** (50.0 mg, 7.4×10^{-6} mmol) was dissolved in 0.2 M HCl (50.0 mL, 10 mmol). The solution was left stirring gently over a period of 24 h. The mixture was dialysed against water using dialysis tubing-Visking size 8 32/32 in. and freeze dried.

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