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Development of the Biocatalytic Resolution of 2-azabicyclo[2.2.1]hept-5-en-3-one as an entry to Single-Enantiomer Carbocyclic Nucleosides

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Abstract: For the resolution of the bicyclic lactam 2-azabicyclo[2.2.1]hept-5-en-3-one, efficient whole cell biocatalysts have been identified and from these, enzymes (lactamases) have been isolated. While the two enzymes obtained act on different enantiomers of the lactam, either can be used in scaleable processes to obtain synthons for carbocyclic nucleosides having the natural configuration.

Carbocyclic nucleosides, where the ribose oxygen of nucleosides has been replaced by a methylene group, have grown in prominence as chemotherapeutic agents for the treatment of viral infections (eg HIV, herpes) and as cardiac vasodilators [1]. A benefit is that the absence of ribosyl oxygen prevents cleavage by nucleases to ribose plus base leading to greater bioavailability. For the purpose as pharmaceutical agents, it is necessary that the carbocyclic nucleosides are in a single enantiomer form and generally this is that corresponding to the natural configuration.

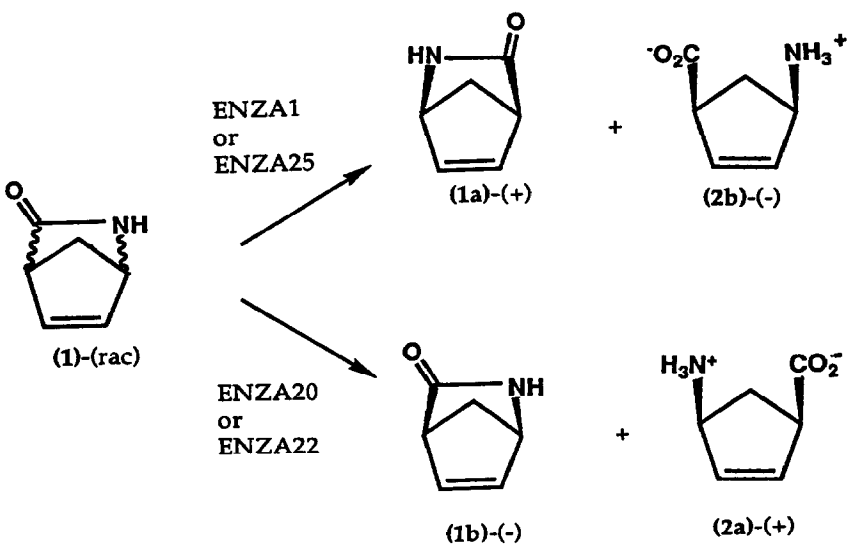
The bicyclic lactam 2-azabicyclo[2.2.1]hept-5-en-3-one (**1**) has, in its racemic form, been shown by the group of Vince[2] to be a versatile synthon for the preparation of carbocyclic nucleosides. While there are several points in the synthetic pathways to the carbocyclic nucleosides where resolution into the enantiomers could be effected, it is most economical to have access to the single enantiomers of the bicyclic lactam. An earlier communication by us described whole cell biocatalytic systems that could effect resolution[3]. In addition, the use of the (-)-enantiomer of the lactam as the precursor of the anti-HIV agent (-)-carbovir has been described in full[4]. Here we describe results obtained with two new enantiocomplementary whole cell biocatalysts, and the lactamase enzymes derived therefrom, on the enantiospecificity of the resolution of 2-azabicyclo[2.2.1]hept-5-en-3-one together with some synthetic chemistry enabling either biocatalyst to be used for the synthesis of natural configuration carbocyclic nucleosides.

RESULTS AND DISCUSSION

Isolation and Identification of Strains

In our earlier work we had identified two microbial strains identified as a *Rhodococcus* sp (termed ENZA1) and *Pseudomonas solanacearum* (ENZA20) which enantiospecifically cleaved the [1(R),4(S)]-(-)- and [1(S),4(R)]-(+) enantiomers of 2-azabicyclo[2.2.1]hept-5-en-3-one respectively (Scheme 1).[3] These microbial strains could be used effectively for the rapid resolution of **(1)** at up to $100g.L^{-1}$ in an aqueous solution to give solutions containing one enantiomer of the bicyclic lactam and the other as the ring-opened amino acid. The resulting processes could be used at the multi-kilogram scale.

As a progression of this, we sought alternative microbial strains with lactamase capability, having improved properties such as stability and degree of selectivity. Several sewage and soil samples were screened, selecting micro-organisms able to utilise N-acetyl-L-phenylalanine as a sole source of carbon, thus only microbes with hydrolytic activity to the amide bond were isolated. Screening of many isolates for their ability to hydrolyse racemic **(1)** in liquid culture, containing salts, carbon source and racemic **(1)**, resulted in two promising strains. One was identified as a *Pseudomonas fluorescens* strain (referred to as ENZA22) and the other tentatively as an *Aureobacterium* species (ENZA25).



Scheme 1 Microbial resolutions of 2-azabicyclo[2.2.1]hept-5-en-3-one **(1)**

Whole Cell Biotransformations with ENZA22 and ENZA25

Both the ENZA22 and ENZA25 strains were readily grown to a biomass of about 3 g/L dry weight by shaken-flask liquid culture on simple media, containing salts, a carbon source (glucose) and yeast extract. Recovery of cells by centrifugation, then resuspension in phosphate buffer (pH7) containing racemic (1) at 10-50g/L allowed a rapid hydrolysis of the lactam to the 4-amino-2-cyclopentene-1-carboxylic acid, which could be monitored by HPLC. In typical whole cell biotransformations, ENZA22 and ENZA25 promoted hydrolysis at similar rates (Figure 1). However, the analysis of the culture filtrate at the end of the biotransformation indicated complementary specificities, where ENZA22 hydrolysed the (+)-enantiomer and ENZA25 the (-)-enantiomer.

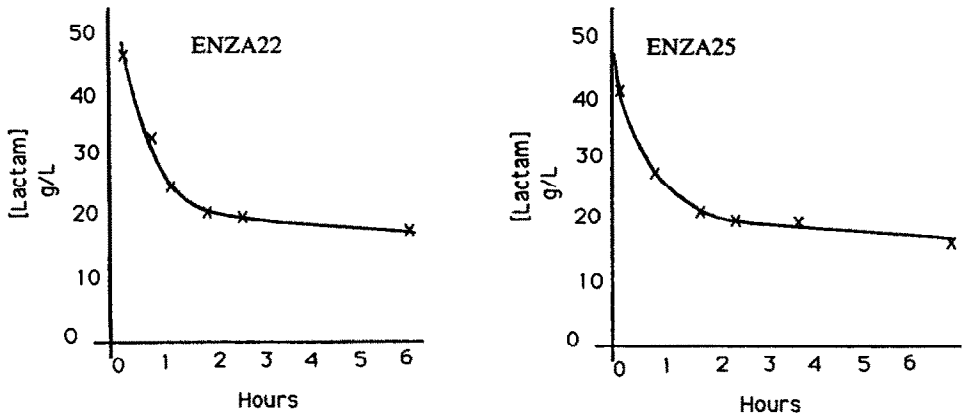
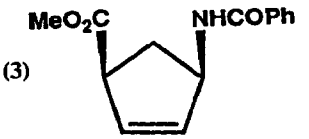


Figure 1 Timecourse of biotransformations of racemic (1) with the ENZA22 and ENZA25 strains.

Enantiomeric Excess Determinations

In developing a suitable measurement of enantiomer excess, we took note of earlier work which attempted to resolve several N-derivatised carboxyesters of the 4-amino-2-cyclopentenecarboxylic acid using various esterases or lipases though with only poor to moderate selectivity[5,6]. This work indicated enantiomeric separation of the N-benzoylamide methyl ester of 4-amino-2-cyclopentenoic acid (3) using the chiral protein based α-AGP HPLC column[5]. In our experience, however, this HPLC column proved unstable over long periods of time, and a much more stable and increased resolution was obtained using the Daicel Chiralcel OJ column. This column also gave good detection limits, where 0.1% enantiomer could be detected under normal conditions.



(3)

Table 1 Enantiomeric excess and determination of E values for ENZA22 and ENZA25 whole-cell resolutions

Strain	ee_s	ee_p	E
ENZA22	0.93	0.93	94
ENZA25	0.97	0.91	76

By appropriate derivation and HPLC assay, ee values could be obtained both for residual lactam and amino-acid product and the results are presented in Table 1. It is seen that enantiospecificity (E) value[7] for both biocatalysts are comparable. Note however, that while enantiospecificity is not perfect, by taking the biotransformations beyond 50% conversion leaves bicyclic lactam that is essentially optically pure.

Purification and Immobilisation of ENZA25 Lactamase

Initially our efforts were to purify and immobilise the lactamase enzyme from the ENZA22 strain since it is this that leaves residual lactam of stereochemistry appropriate for carbocyclic nucleosides of natural configuration. However while the lactamase enzyme could be isolated, we found it to be unstable when isolated from the whole cell environment. Hence, attention was focused on the ENZA25 strain which provided a much more stable and robust lactamase enzyme[8]. Indeed, it retained much of its lactamase activity even when exposed to 70°C for 5-10 minutes. This extent of heat treatment would destroy the majority of activity of many other hydrolase enzymes.

In order to isolate this unique lactamase, ENZA25 was purified from the whole cells by standard methods which are scaleable by a process including ammonium sulphate fractionation and a single anion exchange chromatography step. The resulting lactamase extract was purified 15-fold, and was used directly for immobilisation onto a glutaraldehyde containing polymeric support.' The immobilised lactamase was used to biotransform a buffered aqueous solution of racemic (1) (50g/L), by recirculating the substrate solution over the enzyme support in a continuous cycle. Initial immobilisation of 40 units (1 unit = 1 mg amino acid produced/hour) of lactamase resulted in 60% recovery of activity. The activity of the immobilised enzyme did not decrease upon reuse, and no detectable loss was noted after 20 days. Over a 96 hour biotransformation of 50 ml of an 87 g/L lactam solution, 50% hydrolysis was observed.

At the end of the first biotransformation, analysis of product and substrate ee values gave no detectable enantiomer contamination in either product or substrate, giving an E value[7] (within the limits of detection) of ≥ 7000. Clearly during purification and immobilisation, non-specific hydrolase activities have been removed.

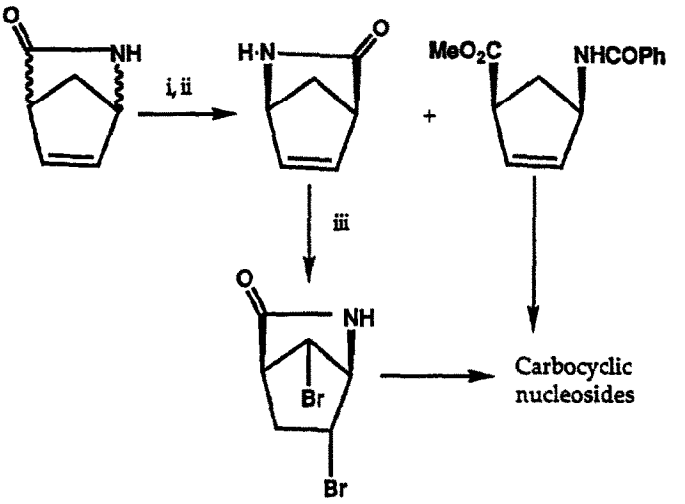
Isolation of Biotransformation Products

Almost quantitative recovery of the unreacted enantiomer of the bicyclic lactam (i.e. **1b**) from the biotransformation with immobilised ENZA25 lactamase was achieved by continuous extraction of the aqueous product solution with dichloromethane for 48 hours, whereafter, concentration of the solvent allows facile crystallisation of the lactam. The lactam recovered from immobilised enzyme biotransformations was noticeably less coloured compared with whole cell biotransformation and required no further purification.

The 4-amino-2-cyclopentenecarboxylic acids (**2a** and **2b**) were conveniently isolated in high yield by derivatisation, either by N-tert-butoxycarbonylation and extraction of the carboxylic acid, or by N-benzoylation (for convenience of the ee assay) followed by formation of the methyl ester. The free amino acids could themselves be recovered by concentration of the aqueous solution after extraction of the residual lactam followed by acetone dilution and in this respect it is noteworthy that the biotransformation naturally produces the amino acid in its neutral form, something less easily achieved by chemical hydrolysis.

While the ENZA25 lactamase biotransformation leaves the bicyclic lactam (**1a**) which would lead to carbocyclic nucleosides of unnatural configuration, the amino acid product is of the correct configuration and its isolation as the benzoylamide methyl ester provides a known intermediate to carbocyclic nucleosides.[5]

An issue concerning resolution processes is of how to utilise the unwanted enantiomer which otherwise merely builds up as waste and limits the theoretical yield to a maximum of 50%. While we can envisage no process for racemisation or inversion of the amino acid (**2a**), an inversion process for the bicyclic lactam (**1**) has been demonstrated when subjected to bromination, when there is a skeletal rearrangement. This has allowed the preparation of either enantiomer of a GABA agonist from both enantiomers of the bicyclic lactam.[9] Thus potentially a process can be envisaged whereby a biotransformation of racemic lactam with immobilised ENZA25 lactamase gives amino acid which is isolated as its ester-amide derivative and recovered bicyclic lactam is inverted via the dibromide or an analogous route, thus providing an enantioconvergence to material of required configuration (Scheme 2).

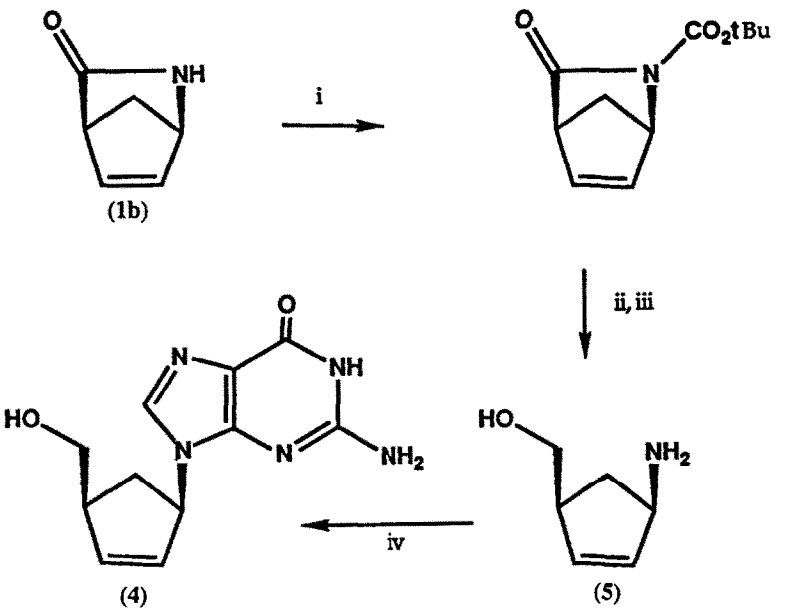


Reagents i ENZA25 lactamase, H_2O; ii PhCOCl, OH^-; iii Br_2, CH_2Cl_2

Scheme 2 Enantioconvergent route to Carbocyclic nucleosides

Other Chemistry leading to Carbocyclic Nucleosides

The established route from the bicyclic lactam (**1**) to the carbocyclic nucleoside carbovir (**4**) is quite lengthy[2,4]. The lactam is first converted to an ester-amide derivative, typically the acetamide methyl ester which is reduced with an activated borohydride such as the calcium salt and the product deacetylated to reach the key aminoalcohol (**5**). This route is used since the lactam is inert to direct reduction by sodium borohydride. However, we found that after t-butoxycarbonylation of the lactam (**1**) on the nitrogen[10], as reported for the N-ethoxycarbonyl derivative[11], reduction is facile and directly leads to the N-BOC protected derivative of the amino alcohol. This provides a simple efficient approach to the carbocyclic nucleosides (Scheme 3).



Reagents i $(tBuO_2C)_2O$, DMAP, NEt_3, CH_2Cl_2; ii $NaBH_4$; iii CF_3CO_2H; iv Known steps

Scheme 3 Route to (-)-Carbovir

CONCLUSION

The work described shows that there is ready access to optically pure synthons that lead to either enantiomer of carbocyclic nucleosides[12]. Work is in progress on optimising the immobilised ENZA-25 lactamase biotransformation for multi-kilogram operation.

EXPERIMENTAL SECTION

Materials

Racemic (1) was prepared from the cycloaddition of cyclopentadiene with tosyl cyanide[13] as described by Daluge and Vince.[2] All other chemicals were from commercially available sources. Proton NMR spectra (200 MHz) in the solvent indicated were recorded on a Bruker AM200 spectrometer. Melting points were measured on an Electrothermal 9200 apparatus. Optical rotations were measured using an Optical Activity PolAar 2001 instrument.

Biocatalyst Production

1) Identification of biocatalysts. Two strains with lactamase activity were isolated by enrichment culture on media containing minimal salts and N-acetyl-L-phenylalanine as a sole carbon source. One (referred to as ENZA22) was identified as *Pseudomonas fluorescens*, and the other (ENZA 25) putatively as an *Aureobacterium* species.

2) Shaken flask growth. Strains were grown in liquid media containing KH_2PO_4 (7g/L), Na_2HPO_4 (2 g/L), $MgSO_4.7H_2O$ (0.4 g/L), $FeCl_3.6H_2O$ (0.02 g/L), $CaCl_2.H_2O$ (0.02 g/L), NH_4Cl (5 g/L), yeast extract (5 g/L) and glucose (5 g/L), adjusted to pH 7. Cells were cultivated at 30°C in conical flasks on an orbital shaker (350 rpm). After 24 hours growth cells were harvested by centrifugation, then resuspended in NaH_2PO_4 buffer (pH 7, 10mM) at an absorbance of 10-20 at 520 nm.

Whole Cell Biotransformations of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one (1)

Racemic 2-azabicyclo[2.2.1]hept-5-ene-3-one (1) was dissolved to 10 g/L in a whole cell suspension of either ENZA22 or ENZA25. The cultures were incubated on orbital shakers (350 rpm) at 30°C in conical flasks. Biotransformations were monitored by HPLC, following the disappearance of (1) and appearance of 4-amino-2-cyclopentenecarboxylic acid (2).

Purification and Immobilisation of ENZA25 Lactamase

1) Cell-free extracts of ENZA25. Cells were grown as described above, then harvested by centrifugation and stored as a frozen paste. Frozen cells were mixed with Tris - HCL buffer (pH 8, 10mM) containing lysozyme (5mg/ml) overnight. Cell debris was then removed by centrifugation after the addition of streptomycin sulphate, giving a particulate free lysate. Further purification was achieved by partial selective precipitation of unwanted proteins by making the extract 45% of saturated with respect to

$(NH_4)_2SO_4$. The precipitate was removed by centrifugation. The resulting supernatant was dialysed to low conductivity (3 mS), then bound to a QA52 anion exchange gel. The lactamase was then eluted by NaCl gradient (0-1M), resulting in a 15 fold purification. The resulting eluate was used in the immobilisation procedure.

2) Immobilisation of lactamase activity. The lactamase solution was applied to glutaraldehyde activated discs (Acti-Disk, FMC Corporation, pine Brook, NJ, USA) by recycling the solution through the disc for 20 hours. A disc of $17cm^2$ was assayed to contain 8 mg protein and 40 units lactamase activity (1 unit = 1 mg amino acid/hour).

Biotransformation of (1) by Immobilised Lactamase

A solution of racemic (**1**) (50 g/L) containing KH_2PO_4 (10mM,pH8) and NaN_3 (0.02%) was passed through the disc containing immobilised lactamase at 2.25ml/min, 22°C, for 16 days, with continuous recycling. The disappearance of (**1**) and appearance of 4-amino-2-cyclopentenecarboxylic acid (**2**) were monitored by HPLC.

Isolation of enantiomers of 2-azabicyclo[2.2.1]hept-5-en-3-one (1a and 1b)

For whole cell biotransformations, cells were first removed by centrifugation then (**1a**) or (**1b**) was continuously extracted from the biotransformation culture into dichloromethane for 48 hours. The dichloromethane solutions were concentrated under reduced pressure, then (**1a**) or (**1b**) recrystallised by addition of n-hexane. The [1(S), 4(R)]-2-azabicyclo[2.2.1]hept-5-en-3-one-(**1a**) had mp 94-95°C; $[\alpha]_D^{25}$ = + 558 (c = 1, CH_2Cl_2), found C, 65.69; H, 6.38; N, 12.95%. C_6H_7NO requires C, 66.03; H, 6.46; N, 12.84%. The [1(R), 4(S)] enantiomer (**1b**) had mp 93-95°C; $[\alpha]_D^{25}$ - 557 (c = 1, CH_2Cl_2), found C, 66.04; H, 6.52; N, 12.72%. Both enantiomers gave ^1H NMR spectra identical to that of the racemate.

Isolation of enantiomers of 4-Amino-2-cyclopentene-1-carboxylic acid (2)

1) As N-tert-Butoxycarbonyl protected amino acids. The aqueous fraction from which (**1**) had been recovered by continuous extraction was adjusted to pH 10 with NaOH (5M). Methanol (1 volume) then di-tert-butyldicarbonate (1.2 equivalents) were added with stirring at room temperature. As the pH decreased, NaOH (5M) was added to maintain pH 10. After alkali consumption ceased, the methanol was evaporated under reduced pressure, the pH was adjusted to 4 using HCl (3M). The N-BOC protected amino acid was extracted 3 times with an equal volume of ethyl acetate. The organic extracts were dried ($MgSO_4$), filtered, then the filtrate partially evaporated under reduced pressure. The product was then recrystallised by addition of n-hexane. The [1(R), 4(S)] isomer gave mp 148°C (decomp.), $[\alpha]_D^{25}$ + 40.3 (c = 2.1 in CH_2Cl_2). The [1(S), 4(R)] isomer gave mp 149°C (decomp.), $[\alpha]_D^{25}$ - 40.3 (c = 2.1 in CH_2Cl_2); δH (D_2O)

1.45 (9H, s, ^tBu), 1.90 (1H, bm, H-5), 2.55 (1H, bm, H-5), 3.50 (1H, bm, H-1), 4.80 (1H, bm, H-4), 5.90 (2H, bs, H-2 and H-3).

2) As N-benzoylcarboxymethyl protected amino acids. The aqueous fraction from which (1) had been recovered was stirred with 1 volume of acetone, and triethylamine (1.1 equivalents). Benzoyl chloride (1.1 equivalents) was then added slowly and after stirring for 2 hours at room temperature, acetone was evaporated under reduced pressure. The resulting solution was adjusted to pH4 using HCl (2M), then the N-benzoyl acid extracted into ethyl acetate (3 times with equal volumes). The organic extracts were dried ($MgSO_4$), then concentrated. The methyl esters were formed by refluxing the product in methanol with 2% w/v H_2SO_4 for 2 hours. After cooling, the acid was neutralised with saturated sodium bicarbonate solution, then the methanol evaporated under reduced pressure. The amide ester was partitioned into ethyl acetate, dried ($MgSO_4$) then concentrated and recrystallised from ethyl acetate and n-hexane. The [1(S), 4(R)] enantiomer had mp 80.5-82°C, $[\alpha]_D^{25}$= +33.6 (c= 2.2, CH_2Cl_2) (ee = 98.8%) while the [1(R), 4(S)]-enantiomer had mp 80-82°C; $[\alpha]_D^{25}$ = -33.6 (c = 2.2, CH_2Cl_2); δH ($CDCl_2$) 2.05 (1H, dt, H-5, J=12Hz,3Hz), 2.50 (1H, dt, H-5, J=12Hz,8Hz), 3.60 (1H, m, H-1), 3.74 (3H, s, OMe), 5.31 (1H, bt, H-4, J=8Hz), 5.95, 6.05 (2H, m, H-2 and H-3), 7.40 - 7.90 (5H, m, Ph), 7.00 (1H, bd, NH)

3) As the free amino acids. The aqueous solution after extraction of the residual lactam following a biotransformation of (1) with ENZA-22 cells was concentrated to 150 g/L and diluted with an equal volume of acetone. [1(R),4(S)]-4-Amino-2-cyclopentene-1-carboxylic acid (**2a**) crystallised, mp >200°C (decomp.) $[\alpha]_D$ + 242 [c = 2 in H_2O) found C, 56.14; H, 7.47; N, 10.99% ($C_6H_9NO_2$ requires c, 56.68; H, 7.13; N 11.02% δH (D_2O) 2.40 (1H, d, J = 14 Hz), 2.80 (1H, dt, J = 14.8 Hz), 3.83 (1H, m, C<u>H</u>CO_2^-), 4.68 (1H, m, C<u>H</u>NH_3^+), 6.32 (1H, m), 6.66 (1H, m). The [1(S), 4(R)]-4-amino-2-cyclopentene-1-carboxylic acid (**2b**) was prepared similarly, Found C, 56.66; H, 7.28; N, 11.18%.

Enantiomeric Excess Determination of 2-azabicyclo[2.2.1]hept-5-ene-3-one (1) and 4-amino-2-cyclopentene-1-carboxylic acids (2)

The lactams and N-tert-butoxycarbonyl protected amino acids were first hydrolysed to the amino acid in 12 M HCl by heating to reflux for 1 hour. After cooling, methanol (10 volumes) was added, and reflux continued for 1 hour. The solution was then neutralised with saturated sodium bicarbonate solution, and methanol evaporated under reduced pressure. Acetone (1 volume) was added, then triethylamine (1.1 equivalents) and benzoylchloride (1.1 equivalents). The solution was then stirred at room temperature for 1 hour. The resulting N-benzoyl carboxymethyl ester was then purified by preparative TLC, eluting with 3:1 dichloromethane: acetone and collecting the second highest running band with UV absorbance. The product was eluted into isopropanol, filtered, then diluted with mobile phase for HPLC analysis. For analysis by chiral HPLC, enantiomers were separated on a Chiralcel OJ column (25cm x 4.6mm, Daicel)

using 90:10 n-heptane: isopropanol as mobile phase, 1ml/min flow rate and detection wavelength 254 nm.

(-)-N-tert-Butyloxycarbonyl-2-azabicyclo[2.2.1]hept-5-en-3-one

(-)-2-Azabicyclo[2.2.1]-hept-5-en-3-one **(1b)** (50g,0.46 mol) was dissolved in dry dichloromethane (50ml). To this solution was added anhydrous triethylamine (51g,0.51 mol) and N,N-4-dimethylaminopyridine (0.01mol equiv, 0.6g) followed by dropwise addition of di-tert-butyldicarbonate (1.2 mol equiv, 120.3g) in dry dichloromethane (200 ml). After 1 hour the solution was extracted twice with 0.3M potassium hydrogen sulphate, water and brine followed by drying and concentration to a crystalline solid which was recrystallised from ethyl acetate and n-hexane. Yield 88.3g, 91%. m.p. 84-86°C, $[\alpha]_D^{25}$ - 189 (c = 0.89, CH_2Cl_2)

Reduction of (-)-N-tert-butyloxycarbonyl-2-azabicyclo[2.2.1]hept-5-en-3-one

(-)-N-tert-butyloxycarbonyl-2-azabicyclo[2.2.1]hept-5-en-3-one (5.0g, 26 mmol) was dissolved in methanol 20ml). Sodium borohydride (1.2 mol equivalents, 1.18g) was added in portions at 5°C. After 2 hours the solution was concentrated and the residue subject to extractive work-up using chloroform and water, 50 ml and 10 ml respectively. The organic layer was washed with saturated sodium chloride dried and concentrated to a pale yellow oil which was purified by flash chromatography on silica eluting with 5% methanol in diethyl ether. The product gave $\delta H(CDCl_3)$ 1.40 (1H, dt, H-5, J=8,4Hz, 1.44 (9H, s, BOC), 2.44 (1H, dt, H-5, J=12,8Hz), 2.83 (1H, m, H4), 3.56,3.67, (2H, 2xdd, CH_2O-, J=12,4Hz), 4.68 (1H, m, H-1), 5.80 (2H, m, H-2 and H-3).

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