The biosynthetic origin of diketopiperazines derived from D-proline

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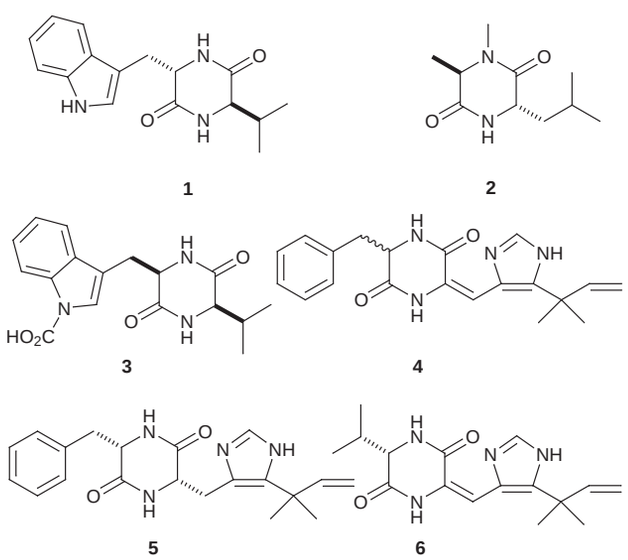
Investigations reveal that the structures assigned to D-α-amino acid containing diketopiperazines (DKPs) 21–25 isolated from the sponge *Calyx* CF. *podatypa* have been reported incorrectly. Synthetic studies and comparison with literature data have enabled the structures of these DKPs to be correctly reassigned. Evidence is presented to suggest that naturally occurring D-Pro-L-Xxx-DKPs are artifacts resulting from non-enzymatic epimerisation of the corresponding L-Pro-L-Xxx-DKP natural products.

Introduction

Diketopiperazines (DKPs) comprise an important class of natural product, many of which are known to exhibit a wide range of biological activity.[1] They occur widely throughout nature and are generally biosynthesised from proteinogenic L-α-amino acids, where cyclisation of a parent L,L-dipeptide affords the core skeleton of the DKP.[2] Further enzymatic functionalisation of this DKP template may then occur to afford more complex natural products.[3] As a consequence of their biosynthetic origin from two L-α-amino acids, most naturally occurring DKPs are *cis*-configured, with over 400 different reports of naturally occurring *cis*-fused L,L-DKPs to date.[4] In contrast to the widespread occurrence of DKPs based on L-Xxx α-amino acids, there are only a small number of reports detailing naturally occurring *cis*- and *trans*-functionalised DKPs derived from non-proteinogenic D-α-amino acids. The biosynthetic origins of these D-α-amino acid containing DKPs are generally unknown, however this class of DKP may be conveniently classified into two different types of DKP depending on whether or not they contain a D-proline residue.

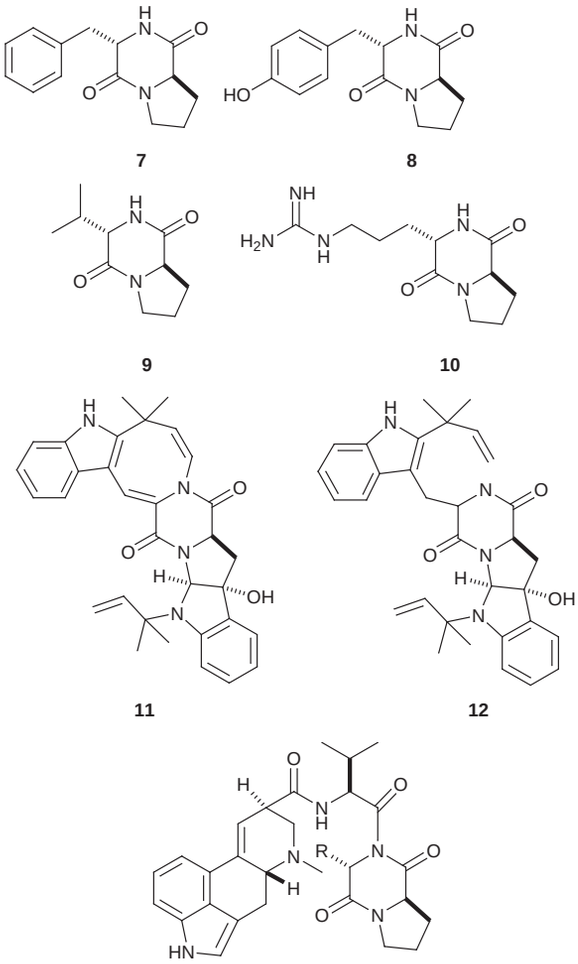
Reports of naturally occurring DKPs which contain D-α-amino acids other than proline are rare, and generally confined to those organisms known to produce the corresponding D-α-amino acid from biosynthesis. *cyclo*-D-Val-L-Trp **1**, for example, was isolated from the fungi *Aspergillus chevalieri*, an organism which is known to biosynthesise D-valine for incorporation into cyclosporins.[5] Similarly, *cyclo*-D-Ala-*N*-methyl-L-Leu **2** was present as a shunt end metabolite from a mutant strain of *Beauveria nivea*. This fungus is deficient in key enzymes involved in cyclosporin biosynthesis; a deficiency which results in the D-Ala it produces being diverted into production of DKP **2**.[6] *cyclo*-*N′*-Carboxy-D-Trp-D-Ile **3** was reported to be isolated from the marine sponge *Rhaphisia pallida*,[7] although the absolute configuration of this DKP **3** is surprising since all other tryptophan derived natural DKPs isolated to date are derived from the isomeric *cyclo*-L-Trp-L-Xxx series.[8] It has been reported that a scalemic DKP, Phenylahistin **4**, which is comprised of a 3:1 mixture of enantiomers in favour of the (*R*)-enantiomer, has been isolated from *Aspergillus ustus*.[9] The origin of the enantiomeric leakage in DKP **4** is unclear, however it is possible that partial epimerisation of a parent DKP **5** occurs prior to the introduction of the exocyclic double bond into **4**. It is interesting to note that the structurally related natural product Aurantiamine **6**, isolated from *Penicillum simplicissimum* in homochiral form, was reported to contain L-valine.[10]

Natural products based on proline DKPs are widespread in nature and the structural complexity and associated biological activity exhibited by this class of DKP is highly impressive.[11]



While the majority of these complex DKPs are derived from L-α-amino acids, a number of D-Pro-derived DKPs also occur, with simple DKPs such as *cyclo*-L-Phe-D-Pro **7**, *cyclo*-L-Tyr-D-Pro **8**, and *cyclo*-L-Val-D-Pro **9** having been isolated from the fungus *Aspergillus flavipes*.[12] *cyclo*-L-Arg-D-Pro **10** is manufactured as a herbicide by culturing the bacteria *Pseudomonas sp*. IZ208,[13] while the structurally more complex insecticides, Okaramines A and C **11**, **12** isolated from *Penicillum simplicissium* AK-40,[14] and the ergopeptam class of fungal alkaloids **13** also contain a D-proline DKP.[15]

Although the biosynthetic origin of the majority of these D-Pro-L-Xxx DKPs is unknown, it is reasonable to assume that they are either derived directly from cyclisation of a parent dipeptide D-Pro-L-Xxx fragment, or they result from epimerisation of a parent L-Pro-L-Xxx based DKP. While proline racemases which produce D-proline have been isolated and characterised,[16] the number of organisms reported to produce these enzymes is limited. Conversely, it is known that equilibration of proline derived *cis*-Pro-Xxx-DKPs under either mild basic or acidic conditions results in formation of the thermodynamically more stable *trans*-Pro-Xxx-DKP diastereoisomers.[17] Furthermore, it has been demonstrated that epimerisation of L-Pro-DKPs under mildly basic conditions is selective,[18] since treatment of *cyclo*-L-Pro-L-Ala **14** with 0.01 M NaOH in MeOH results in exclusive epimerisation at the proline ring junction to afford *cyclo*-D-Pro-L-Ala **15** as the major diastereoisomer.[19] These observations led us to propose that natural products derived from D-Pro-L-Xxx-DKPs are



R derived from Phe, Leu, Ile, Val, homoAla

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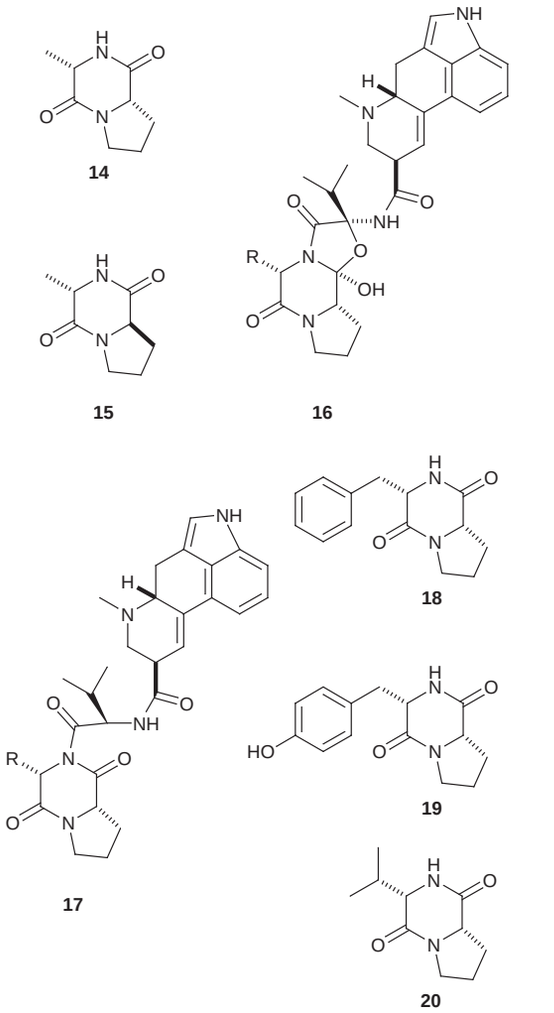
in fact artifacts resulting from non-enzymatic epimerisation of the corresponding parent L-Pro-L-Xxx DKP natural products. Whether this L-proline to D-proline epimerisation process occurs *in vivo*, or during the isolation procedure, is open to debate; however there is evidence that epimerisation of Pro-DKPs may occur under physiological conditions. The ergopeptam class of alkaloids **13** differs from the open chain precursors of the ergopeptine class of alkaloids **16** only by virtue of a D-proline residue in place of an L-proline residue. A biosynthetic pathway has been proposed to explain the occurrence of this class of alkaloid which invokes a parent L-Pro-L-Xxx intermediate **17** that has two possible metabolic fates; enzymatic cyclisation to afford the *cyclo*-ergopeptine series of alkaloids **16**, or irreversible epimerisation *via* a non-enzymatic pathway, to afford the ergopeptam series of alkaloids **13**.[15] Further evidence in support of a non-enzymatic epimerisation pathway to D-Pro-DKPs arises from the observation that the simple D-proline containing DKPs, **7**, **8**, **9** were co-isolated from *Aspergillus flavipes* with the corresponding L,L-DKP diastereoisomers **18**, **19**, **20**.[12]

Since all chemical evidence suggested that epimerisation of proteinogenic *cis*-L-proline derived DKPs will only occur at the α-centre of the proline ring, we were surprised to discover reports on the isolation of naturally occurring DKPs based on D-Pro-D-Xxx and L-Pro-D-Xxx skeletons. The existence of this class of DKP would cast serious doubts on the validity of our epimerisation hypothesis, therefore we closely examined the spectroscopic data cited in evidence for the structure of these DKPs. *cyclo*-D-Pro-D-Leu **21** was reported to be isolated from the blue stain fungi *Ceratocystis clavigera*, with a specific rotation of −102 (*c* 0.25, $CHCl_3$),[20] however this assignment

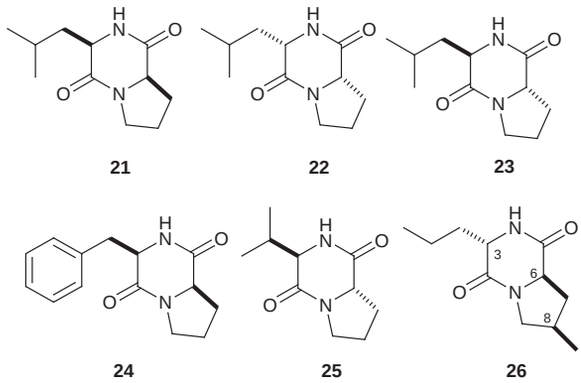


was clearly incorrect since the negative sign of rotation revealed this compound to be the enantiomer *cyclo*-L-Pro-L-Leu **22** {lit., $[\alpha]_D^{23}$ −136 (*c* 0.12, EtOH)}.[21] Some confusion exists surrounding the structure of the maculosins, a family of proline based DKPs isolated from fungal sources. While this class of compound was correctly assigned as containing an L-proline and an L-α-amino acid, their structures were drawn incorrectly as the corresponding D-Proline-D-Xxx diastereoisomers;[22] a mistake which has been compounded by subsequent authors reporting on this class of DKP.[23]

Finally, Adamzceski, Reed and Crews[24] reported on the isolation and characterisation of a number of DKPs from the sponge *Calyx* CF. *podatypa* including *cyclo*-D-Pro-D-Leu **21**, *cyclo*-L-Pro-D-Leu **23**, *cyclo*-D-Pro-D-Phe **24**, *cyclo*-L-Pro-D-Val **25** and *cyclo*-L-norvaline-(4*R*)-methyl-D-Pro **26**.[24,25] We believed these assignments to be unsafe since the structures of these DKPs **21**, **23**–**25** would require them to be derived from a pool of D-α-amino acids. Even more remarkably, the novel DKP **26** was reported to contain two non-proteinogenic α-amino acids, L-norvaline and (4*R*)-methyl-D-proline, both of which occur very rarely in nature.[26] After carrying out a survey of the literature, in conjunction with related synthetic studies, we now report that the structures of DKPs **21**, **23**–**26** are incorrect, and have reassigned the structures of these natural products.

Results and discussion

Our initial studies centred on the structure of DKP **26**. Adamzceski, Reed and Crews[24] reported that this unusual DKP was comprised of two non-proteinogenic α-amino acids, L-norvaline and (4*R*)-methyl-D-proline, however close examination of the spectroscopic and physical data cited in evidence for its gross structure revealed a number of discrepancies that led us to



believe that the proposed structure was incorrect. The major points of concern over this structure were:

1) The ^{1}H NMR spectroscopic data reported for DKP **26** were inconsistent with the reported structure. The H_9 geminal ring protons at δ 3.69 and δ 3.53 had no common geminal coupling constant. They were both assigned as a doublet of triplets with coupling constants of 8.3 Hz and 3.9 Hz and 10.4 Hz and 2.1 Hz respectively! A similar discrepancy was observed for the DKP ring protons H_3 at δ 3.77 and H_6 at δ 4.08 where the transannular coupling constant was reported as 1.8 Hz for H_3 and 3.0 Hz for H_6.

2) The absolute and relative stereochemistry at the DKP ring junction was assigned as L-C_3 and D-C_6 by comparing the positive sign of rotation of the natural product with that of a series of known L-proline DKPs of negative rotation. As clearly stated by the authors:[24] '*The overall sign of* $[\alpha]_D$ *for DKPs in this study is either negative or positive depending only on the Pro absolute configuration being* S *or* R, *respectively. In addition the actual magnitude of this* $[\alpha]_D$ *varies from* −*134 to* −*185° for the* S-*Pro*-S-*Xxx substances versus* −78 to −120° for the S-*Pro*-R-*Xxx compounds*, *where Xxx has an aliphatic side chain of three carbons or more.*'

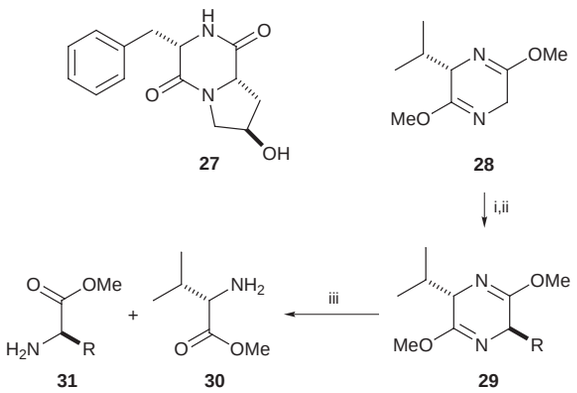
3) The use of comparative specific rotations to assign the absolute stereochemistry of related structures is suspect since small changes in the conformation of structurally related compounds can lead to large changes in specific rotation. If we accept that a position rotation indicates that the proline ring junction is *R*-configured (D *vide-infra*) and consider the relative stereochemistry across the DKP ring, it is clear that the rotation reported for DKP **26** of +128 lies between the arbitrary boundaries proposed by the authors for *cis*- and *trans*-proline DKPs. Furthermore the fundamental premise on which this analysis is based is flawed, since the authors themselves have reported that the structurally related DKP *cyclo*-L-phenylalanine-*trans*-hydroxy-L-proline **27**, isolated from *Jaspidea* sponge has a relatively small rotation of −6.7 (*c* 0.016, MeOH), which lies well outside the cited specific rotation values for *cis*- and *trans*-proline DKPs![27]

4) No clear spectroscopic evidence was presented to justify the assertion that the stereochemistry at the C_8-methyl of **26** was *R*-configured.

In order to resolve the uncertainties surrounding the structure of this natural product we have prepared the proposed DKP **26** using an approach based on a variation of Schöllkopf's bis-lactim ether methodology.[28]

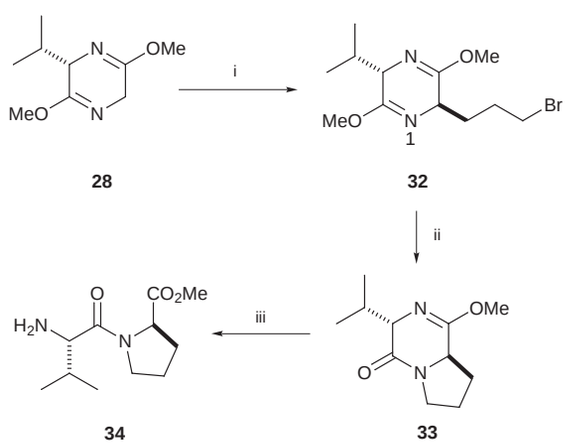
Synthetic approach to DKP 26

Schöllkopf's auxiliary **28** has been widely used for the preparation of homochiral non-proteinogenic α-amino acids. Treatment of the lithium anion of **28** with a wide range of electrophiles results in *trans*-alkylated auxiliaries **29** in good de: subsequent hydrolysis of **29** with 0.1 M HCl affords a mixture of homochiral valine **30** and the desired α-amino acid **31** as their methyl esters which may be easily separated by distillation (Scheme 1).[28]



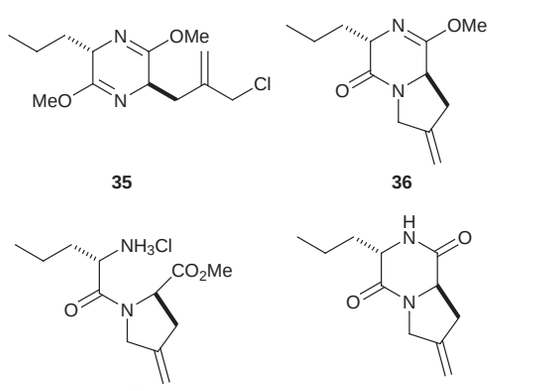
Scheme 1 *Reagents and conditions*: i, Bu^{n}Li, THF, −78 °C; ii, RX; iii, 0.1 M HCl

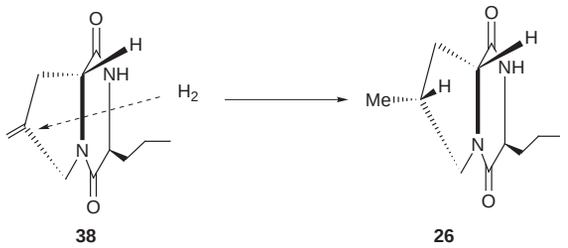
Alkylation of the lithium anion of auxiliary **28** with excess 1,3-dibromopropane, for example, afforded *trans*-alkylated auxiliary **32** in >90% de. Treatment of bromide **6** with sodium iodide in refluxing acetone resulted in cyclisation and deprotection of the N_1 lactim ether bond *in situ*, to afford proline mono-lactim **33**. Mild acid catalysed hydrolysis of **33** resulted in selective cleavage of the lactim ether bond, enabling selective preparation of proline derived dipeptides **34** in good yield (Scheme 2).[29]



Scheme 2 *Reagents and conditions*: i, Bu^{n}Li, THF, −78 °C; 1,3-dibromopropane; ii, NaI, acetone, Δ; iii, 0.1 M HCl

We wished to employ this cyclisation/deprotection methodology for the asymmetric synthesis of DKP **26**. Central to our retrosynthetic analysis was the preparation of *trans*-alkylated dihydropyrazine **35** which we proposed would be readily cyclised to mono-lactim **36**. Subsequent cleavage of the lactim ether bond of **36** followed by careful recyclisation of the resulting dipeptide **37** would provide a substrate **38** ideally suited for the introduction of the new stereocentre at C_8.





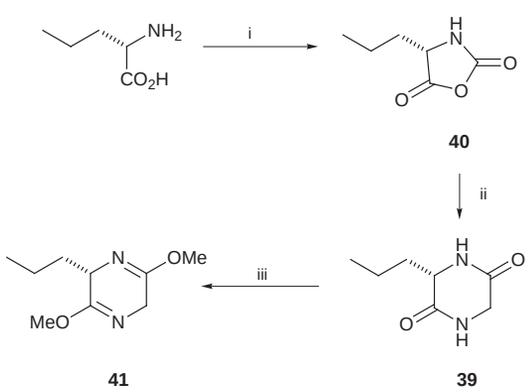
H_2 delivered from least hindered *exo* face

Fig. 1

Molecular modelling of the conformation of DKP **38** indicated that hydrogenation of the exocyclic olefin bond would occur under steric control where hydrogen would be delivered from the exocyclic face of bicyclic DKP **38** to afford DKP **26** with the desired (*R*)-*endo*-methyl stereochemistry at C_8 (Fig. 1).[30]

Synthesis of *cyclo*-[L-norvaline-(4*R*)-methyl-D-proline] 26

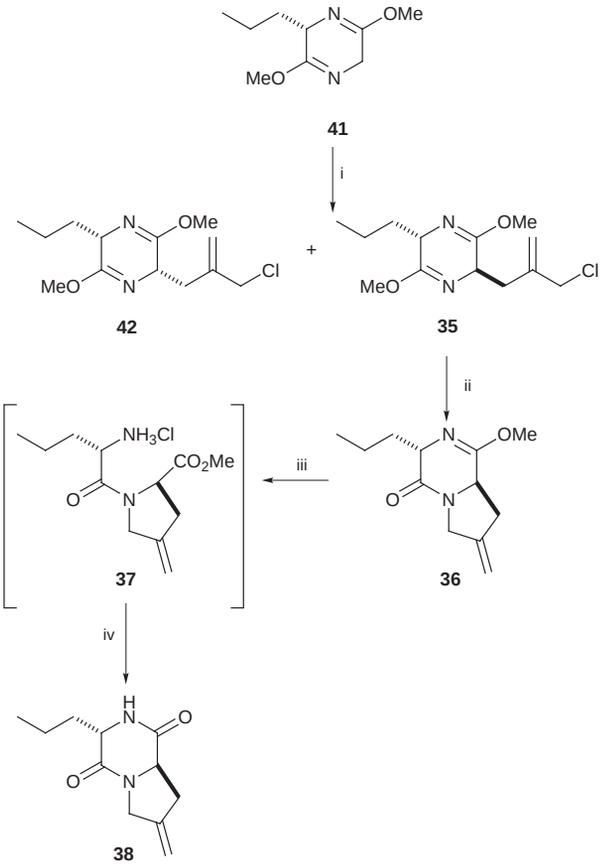
DKP **39** was prepared according to the literature procedure described for Schöllkopf's auxiliary **28** *via* condensation between (*S*)-norvaline-Leuch's anhydride **40** and glycine methyl ester, and subsequently protected as its bis-lactam ether **41** by treatment with Me_3OBF_4 in dichloromethane (Scheme 3).[31]



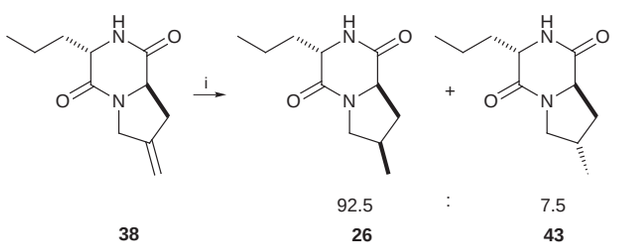
Scheme 3 *Reagents and conditions*: i, $COCl_2$, THF, PhMe, 50 °C; ii, glycine methyl ester·HCl, triethylamine, $CHCl_3$, THF, −78 °C to −20 °C then PhMe, Δ; iii, Me_3OBF_4, CH_2Cl_2

Deprotonation of bis-lactim ether **41** with Bu^{n}Li in THF at −78 °C, followed by alkylation with 3 equivalents of methallyl dichloride afforded a mixture of *trans*-alkylated **35** and *cis*-alkylated **42** in 90% de, which was purified by chromatography to give diastereomerically pure *trans*-**35** in 64% yield and *cis*-**42** in 3% yield. The major diastereoisomer **35** was smoothly cyclised to its bicyclic mono-lactim ether **36** by treatment with sodium iodide in refluxing acetone. Selective hydrolysis of the lactim ether bond of **36** with 0.1 M HCl gave dipeptide **37** which was not isolated but immediately cyclised under non-epimerising conditions (*N*-methylmorpholine, acetic acid in butan-2-ol)[32] to afford DKP **38** in an overall 53% yield from bis-lactim ether **41** (Scheme 4).

Hydrogenation over palladium on carbon of the exocyclic double bond of DKP **38** occurred in 85% de to afford a mixture of the (8*R*) and (8*S*) diastereoisomers **26** and **43** (Scheme 5). The major diastereoisomer **26** was purified by fractional recrystallisation of the minor diastereoisomer **43** from ethyl acetate and the absolute configuration of the new methyl group of DKP **26** confirmed as 8*R* by selected ^{1}H NMR NOE experiments (Fig. 2). The diastereofacial selectivity observed for hydrogenation of DKP **38** was consistent with the proposed model where hydrogen is delivered from the *exo*-face of the concave bicycle as shown in Fig. 1.



Scheme 4 *Reagents and conditions*: i, Bu^{n}Li, THF, −78 °C then $H_2C{=}C(CH_2Cl)_2$, −78 °C to RT; ii, NaI, acetone; iii, 0.1 M HCl; iv, *N*-methylmorpholine, acetic acid, butan-2-ol



Scheme 5 *Reagents and conditions*: i, 5 mol% Pd–C, 1 atm. H_2, MeOH

Revision of the structure of the natural DKPs 21, 23–26

With an authentic sample of DKP **26** in hand we compared its spectroscopic data with the spectroscopic information published by Adamzceski, Reed and Crews[24] for the proposed natural product **26** which clearly revealed that this structure was incorrect. Close examination of the *original* spectroscopic data[33] strongly suggested a compound containing a DKP skeleton containing the two α-amino acids, isoleucine and proline. Since Adamzceski, Reed and Crews had already reported on the isolation of *cis*-fused *cyclo*-L-Ile-L-Pro **44** from *Calyx* CF. *podatypa*,[24] it seemed reasonable to us that the reported spectroscopic data were consistent with *trans*-fused *cyclo*-L-Ile-D-Pro **45**. This prediction was confirmed by formal synthesis of DKP **45** from L-isoleucine and D-proline (Scheme 6). DKP **45** gave ^{1}H, ^{13}C, and COSY NMR spectra which were identical to those acquired by Adamzceski, Reed and Crews for the natural product **26**, while a positive optical rotation of $[\alpha]_D^{23}$ +98 (*c* 0.1, EtOH), [lit.,[24] DKP **26** reported as $[\alpha]_D^{23}$ +128 (*c* 0.1, EtOH)] enabled the absolute configuration of the natural product to be confirmed as *cyclo*-L-Ile-D-Pro **45**.

Having clearly determined that the structure of **26** was incorrect our attention then turned to the structures assigned to

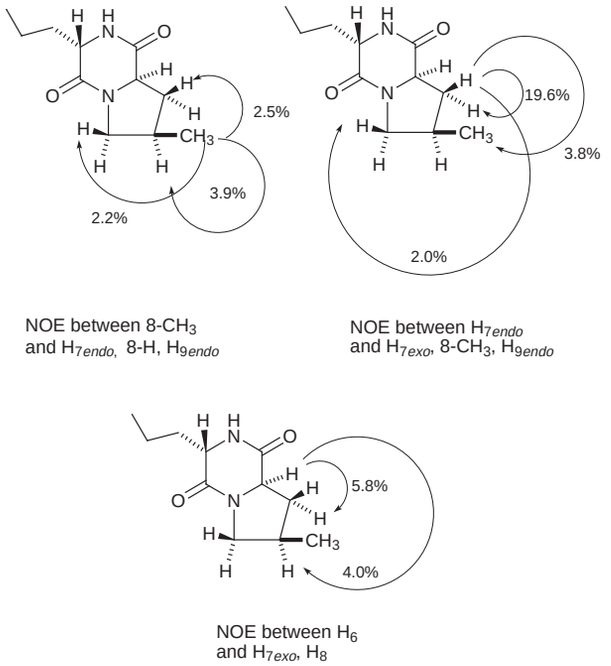
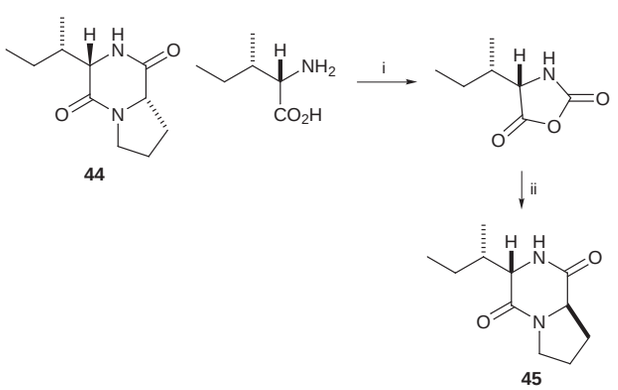


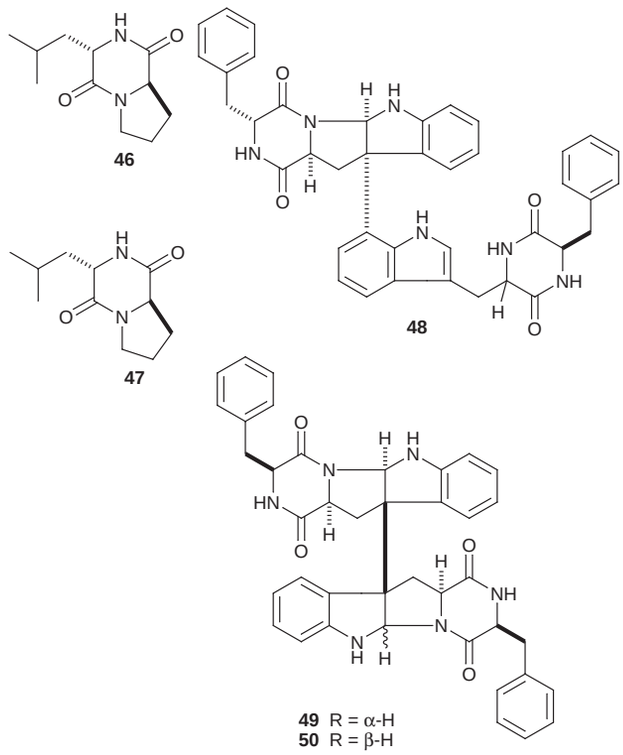
Fig. 2



Scheme 6 *Reagents and conditions*: i, $COCl_2$, THF, PhMe, 40 °C; ii, (*R*)-proline methyl ester, $CHCl_3$, THF, −78 °C to −20 °C; EtOH, Δ

the simple D-proline containing DKPs *cyclo*-D-Pro-D-Leu **21**, *cyclo*-L-Pro-D-Leu **23**, *cyclo*-D-Pro-D-Phe **24**, *cyclo*-L-Pro-D-Val **25**. After close examination of the reported ^{1}H, ^{13}C NMR spectroscopic data/specific rotation data, and comparison with reported literature data for authentic synthetic samples, we deduced that the structures of DKPs **21**, **23**–**25** had also been reported incorrectly, and that they should be reassigned to the known DKPs *cyclo*-D-Pro-L-Leu **46**,[34] *cyclo*-L-Pro-L-Leu **22**,[35,36] *cyclo*-D-Pro-L-Phe **7**[36,37] and *cyclo*-L-Pro-L-Val[36,38] **20** respectively.

In light of these observations we suspect that the structure of a recently isolated dimeric DKP **48** from *Aspergillus niger*,[39] reported to be comprised from two D-phenylalanine units, and two L-tryptophan residues may also be incorrect. Bearing in mind the ease of epimerisation of Pro-DKPs we suggest that this natural product is more likely to be derived from two L-phenylalanine residues and two L-tryptophan residues one of the latter having undergone an inversion *via* the proline-DKP pathway described above. This assertion is substantiated by the isolation of two other structurally related isomers **49**, **50** from *Aspergillus* species,[40] both of which contain L-phenylalanine residues, and the fact that no D-phenylalanine could be incorporated into DKP **48** during directed biosynthetic studies.[39]



Conclusion

To conclude, we have demonstrated that the structures assigned to the naturally occurring DKPs **21**, **23**–**26** from *Calyx* CF. *podatypa* were reported incorrectly and have reassigned the structures of these natural products to the known DKPs **46**, **22**, **7**, **20** and **45** respectively. This reinvestigation clearly highlights the dangers in assigning formal structures to natural products in the absence of rigorous spectroscopic proof. These studies also demonstrate that the practice of assigning the absolute stereochemistry of a natural product by simple comparison of the sign and magnitude of its specific rotation with structurally related analogues of known rotation/absolute configuration should be actively discouraged. In light of these observations we have been able to present significant evidence to suggest that natural products derived from D-Pro-L-Xxx-DKPs are in fact artifacts which result from non-enzymatic stereospecific epimerisation of parent L-Pro-L-Xxx DKP natural products.

Experimental

Melting points (mp) were obtained using a Thermogalen III™ or Griffin Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter with a thermally jacketted 10 cm cell at approximately 20 °C. Concentrations (*c*) are given in g 100 ml^{-1}. Infrared (IR) spectra were recorded as KBr discs on a Perkin-Elmer 1750 Fourier Transform spectrometer. Absorptions are reported in wavenumbers (cm^{-1}). Proton magnetic resonance spectra (^{1}H NMR) were recorded at 200 MHz on a Varian Gemini 200 or a Bruker AC200 spectrometer, at 300 MHz on a Bruker WH300, at 400 MHz on a Bruker AC400 and at 500 MHz on a Bruker AM500 spectrometer and are referenced to the residual solvent peak. The following abbreviations were used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. Coupling constants (*J*) were recorded in hertz to the nearest 0.5 Hz. Carbon magnetic resonance spectra (^{13}C NMR) were recorded at 50.3 MHz on a Varian Gemini 200 or Bruker AC200 spectrometer, at 100.6 MHz on a Bruker AC400 spectrometer and at 125.7 MHz on a Bruker AMX500 spectrometer using DEPT editing. Diastereomeric excesses

were determined by peak integration of the crude reaction products' ^{1}H NMR spectrum. Low resolution mass spectra *(m/z)* were recorded on a VG Micromass ZAB 1F, a VG Masslab 20-250, a GCMS Trio 1, a VG BIO Q or a APCI Platform spectrometer, with only molecular ions (M^+), fragments from molecular ions and major peaks being reported. Microanalyses were performed by Mrs V. Lamburn or Mr R. Prior, Dyson Perrins Laboratory, University of Oxford. Column chromatography was performed on silica gel (Kieselgel 60). Anhydrous THF was obtained by distillation from sodium/benzophenone ketyl under nitrogen. Petrol refers to light petroleum (bp 40–60 °C), redistilled before use. Ether refers to diethyl ether. Unless otherwise stated all reactions were performed and worked-up under a nitrogen atmosphere.

(*S*)-4-Propyloxazolidine-2,5-dione 40

Phosgene (26.5 ml, 1.93 M solution in toluene, 51.2 mmol) was added to a rapidly stirred suspension of L-norvaline (5 g, 42.7 mmol) in THF (60 ml) at 50 °C. After stirring for 4 h, excess phosgene was removed by bubbling nitrogen through the solution and venting the exhaust gases through saturated aqueous NaOH. The solvent was removed in vacuum to afford a crude product which was recrystallised from ether–petrol (1:1) to afford the title compound as a white crystalline solid (4.27 g, 29.8 mmol, 70%); $[\alpha]_D^{23}$ −37.7 (*c* 1.02, $CHCl_3$); ν_{max}(KBr)/cm^{-1} 1816 (O–C=O), 1757 (N–C=O); δ_H($CDCl_3$, 200 MHz) 1.01 (3H, t, *J* 7.4, 8-CH_3), 1.49 (2H, m, 7-CH_2), 1.84 (1H, m, 6-H_a), 1.94 (1H, m, 6-H_b), 4.35 (1H, t, *J* 5.1, 4-CH), 5.76 (1H, br s, NH); δ_C($CDCl_3$, 50 MHz) 13.3 (8-CH_3), 18.0 (7-CH_2), 33.5 (6-CH_2), 57.6 (4-CH), 153.7 (5-C), 170.5 (2-C).

(*S*)-3-Propylpiperazine-2,5-dione 39

Triethylamine (5.88 g, 58.2 mmol) was added to a stirred solution of glycine methyl ester hydrochloride (3.65 g, 29.1 mmol) in chloroform (30 ml) and the reaction vessel cooled to −78 °C under an atmosphere of nitrogen. (*S*)-4-Propyloxazolidine-2,5-dione **40** (4.16 g, 29.1 mmol) in THF (20 ml) was added dropwise over a period of 30 min and the resultant mixture stirred at −78 °C for 4 h. The reaction mixture was warmed to room temperature, filtered through Celite, and the solvent removed in vacuum to afford dipeptide NH_2-L-norvalgly-OMe as an unstable oil. This oil was refluxed in toluene overnight, the crude solid filtered off, powdered, and dried in vacuum at 50 °C overnight to yield the title compound as a white powder (3.46 g, 22.2 mmol, 76%); $[\alpha]_D^{23}$ +10.1 (*c* 1.00, MeOH); ν_{max}(KBr)/cm^{-1} 3210 (N–H), 1720 (C=O); δ_H(D_2O, 200 MHz) 0.82 (3H, t, *J* 7.4, 9-CH_3), 1.28 (2H, m, 8-CH_2), 1.67 (1H, m, 7-H_a), 1.76 (1H, m, 7-H_b), 3.88 (1H, dd, *J* 18.4, 1.2, 6-H_a), 4.03 (1H, td, *J* 18.4, 1.2, 6-H_b), 4.03 (1H, tt, *J* 5.5, 1.2, 3-CH); δ_C(50 MHz; D_2O) 12.6 (9-CH_3), 16.6 (8-CH_2), 34.9 (7-CH_2), 43.5 (6-CH_2), 54.2 (3-CH), 168.6 (C=O), 171.0 (C=O); *m/z* (M + 1, $APCI^+$) 157 (100%); $C_7H_{12}N_2O_2$ requires *m/z* 156.0899. Found 156.0901.

(*S*)-3-Propyl-2,5-dimethoxy-3,6-dihydropyrazine 41

Me_3OBF_4 (2.90 g, 19.57 mmol) was added to a suspension of DKP **39** (1.02 g, 6.53 mmol) in dry DCM (15 ml), and the reaction mixture stirred for 4 days at room temperature under an atmosphere of nitrogen. The heterogeneous reaction mixture was poured slowly into a large excess of saturated aqueous $NaHCO_3$ (ensuring pH remained >7.5), extracted with fresh DCM (3 × 15 ml), dried ($MgSO_4$), and concentrated in vacuum to afford a crude oil which was purified by chromatography [silica, petrol–ether (7:3)] to yield the title compound as a colourless oil (727 mg, 3.95 mmol, 60%); $[\alpha]_D^{23}$ +92.0 (*c* 0.96, $CHCl_3$); ν_{max}(neat)/cm^{-1} 1694 (C=N); δ_H($CDCl_3$, 300 MHz) 0.92 (3H, t, *J* 7.3, 9-CH_3), 1.22–1.40 (2H, m, 8-CH_2), 1.58–1.82 (2H, m, 7-CH_2), 3.69 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 4.02 (1H, m, 6-CH_2), 4.09–4.16 (1H, m, 3-CH); δ_C($CDCl_3$, 50 MHz) 13.5 (9-CH_3), 17.7 (8-CH_2), 36.2 (7-CH_2), 46.2 (6-CH), 52.2 (2 × OCH_3), 55.5 (3-CH), 162.2 (C=N), 165.3 (C=N); *m/z* ($APCI^+$) 185 (M + 1, 100%); $C_9H_{17}N_2O_2$ requires 185.1290. Found 185.1291.

(3*S*,6*R*)-6-(2′-Chloromethylprop-2′-enyl)-3-propyl-2,5-dimethoxy-3,6-dihydropyrazine 35 and (3*S*,6*S*)-6-(2′-chloromethylprop-2′-enyl)-3-propyl-2,5-dimethoxy-3,6-dihydropyrazine 42

Bu^{n}Li (1 equiv. in hexane) was added to a rapidly stirred solution of dihydropyrazine **41** (368 mg, 2.0 mmol) in dry THF (25 ml) at −78 °C, and the reaction mixture stirred for 15 min. Methallyl dichloride (1.0 g, 8 mmol) was then added dropwise and the reaction mixture stirred at −78 °C for 8 h. The reaction mixture was allowed to warm to room temperature, quenched with aqueous NH_4Cl, dried ($MgSO_4$) and solvent removed in vacuum. Excess methallyl dichloride was removed under high vacuum to afford a mixture of *trans*- and *cis*-diastereoisomers [424 mg, 1.56 mmol, 78%, 90% de judged by interaction of signals at δ 5.02 (major) and δ 5.05 (minor), δ 5.21 (major) and δ 5.23 (minor), in the 500 MHz ^{1}H NMR spectra]. The mixture was separated by chromatography [ether–petrol (1:9)] to afford *trans*-**35** (348 mg, 1.27 mmol, 64% yield) and *cis*-**42** (16 mg, 0.06 mmol, 3%) as light yellow oils.

***trans*-35.** $[\alpha]_D^{23}$ −17.0 (*c* 1.0, acetone); ν_{max}(neat)/cm^{-1} 1693 (C=N); δ_H($CDCl_3$, 500 MHz) 0.91 (3H, t, *J* 7.3, 9-CH_3), 1.22–1.33 (2H, m, 8-CH_2), 1.59–1.80 (2H, m, 7-CH_2), 2.51 (1H, dd, *J* 14.0, 7.5, 1′-H_a), 2.77 (1H, dd, *J* 14.0, 4.3, 1′-H_b), 3.67 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 4.04 (1H, td, *J* 6.3, 4.0, 3-CH), 4.11 (2H, J_{AB} 12.2, δ_{AB} 0.015, 2′-CH_2Cl), 4.16 (1H, m, 6-CH), 5.02 (1H, s, 3′-H_a), 5.21 (1H, d, *J* 1.0, 3′-H_b); δ_C($CDCl_3$, 50 MHz) 13.9 (9-CH_3), 17.8 (8-CH_2), 36.3 (7-CH_2), 37.1 (1′-CH_2), 48.8 (CH_2Cl), 52.31 (6-CH), 52.38 (2 × OCH_3), 55.5 (3-CH), 117.3 (3′-CH_2), 142.3 (2′-C), 162.7 (C=N), 164.2 (C=N); *m/z* ($APCI^+$, M + 1) 275 (33%, $C_{13}H_{22}{}^{37}ClN_2O_2$), 273 (100%, $C_{13}H_{22}{}^{35}ClN_2O_2$); $C_{13}H_{22}{}^{35}ClN_2O_2$ requires 273.1370. Found 273.1370.

***cis*-42.** $[\alpha]_D^{23}$ +31.0 (*c* 1.1, acetone); ν_{max}(neat)/cm^{-1} 1693 (C=N); δ_H($CDCl_3$, 500 MHz) 0.94 (3H, t, *J* 7.7, 9-CH_3), 1.37–1.42 (2H, m, 8-CH_2), 1.47–1.56 (1H, m, 7-CH_a), 1.73–1.82 (1H, m, 7-CH_b), 2.41 (1H, dd, *J* 14.0, 8.6, 1′-H_a), 2.77 (1H, dd, *J* 14.0, 4.4, 1′-H_b), 3.65 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 4.04 (1H, td, *J* 6.3, 4.2, 3-CH), 4.11–4.20 (3H, m, 2′-CH_2Cl and 6-CH), 5.05 (1H, s, 3′-H_a), 5.23 (1H, d, *J* 1.0, 3′-H_b); δ_C($CDCl_3$, 50 MHz) 14.1 (9-CH_3), 18.8 (8-CH_2), 37.6 (7-CH_2), 38.7 (1′-CH_2), 48.6 (CH_2Cl), 52.4 (6-CH and 2 × OCH_3), 55.8 (3-CH), 117.2 (3′-CH_2), 142.2 (2′-C), 163.0 (C=N), 164.9 (C=N); *m/z* ($APCI^+$, M + 1) 275 (33%, $C_{13}H_{22}{}^{37}ClN_2O_2$), 273 (100%, $C_{13}H_{22}{}^{35}ClN_2O_2$).

(3*S*,6*R*)-3-Propyl-5-methoxy-8-methylene-1,4-diazabicyclo-[4.3.0]nonan-2-one 36

Bis-lactim ether **35** (300 mg, 1.1 mmol) and sodium iodide (1.65 g, 11 mmol) were refluxed in acetone (20 ml) for 12 h. The solvent was removed in vacuum and the resulting slurry partitioned between ether (3 × 10 ml) and aqueous sodium thiosulfate (10 ml). The combined organic fractions were dried ($MgSO_4$), and the solvent removed in vacuum to afford the title compound as a colourless gum (231 mg, 1.04 mmol, 94%); $[\alpha]_D^{23}$ +72.0 (*c* 0.5, acetone); ν_{max}(neat)/cm^{-1} 1686 (C=N), 1654 (C=O); δ_H($CDCl_3$, 500 MHz) 0.94 (3H, t, *J* 7.3, CH_3), 1.37–1.49 (2H, m, C$H_2$$CH_3$), 1.63–1.72 (1H, m, C$H_a$$H_b$$CH_2CH_3$), 1.73–1.84 (1H, m, CH_aH_b, CH_2CH_3), 2.63 (1H, m, 7-H_a), 2.77 (1H, ddd, *J* 14.2, 6.4, 1.0, 7-H_b), 3.74 (3H, s, OCH_3), 3.84 (1H, dd, *J* 16.5, 2.0, 9-H_a), 4.15–4.20 (2H, m, 3-H and 6-H), 4.46 (1H, d, *J* 16.5, 9-H_b), 5.09 (1H, s, 8-C=C$H_a$$H_b$), 5.14 (1H, s, 8-C=C$H_a$$H_b$); δ_C($CDCl_3$, 50 MHz) 13.7 (CH_3), 18.5 ($CH_2CH_2CH_3$), 36.7 and 36.8 ($CH_2CH_2CH_3$ and 7-CH_2), 48.9 (9-CH_2), 53.2 (OMe), 55.9 (3-CH), 61.6 (6-CH), 117.3 (8-C=CH_2), 141.3 (8-C), 160.3 (C=N), 169.3 (C=O).

(3*S*,6*R*)-3-Propyl-8-methylene-1,4-diazabicyclo[4.3.0]nonane-2,5-dione 38

Mono-lactim ether **35** (150 mg, 0.72 mmol) was dissolved in 0.1 M HCl (2 ml) and left to stand for 5 min and the solvent removed in vacuum. The gummy residue was immediately dissolved in a solution of *N*-methylmorpholine (200 mg) in butan-2-ol (10 ml) and acetic acid (1 ml) and the reaction mixture heated under reflux for 8 h. The solvent was removed in vacuum to afford a crude oil which was purified by chromatography (silica, ethyl acetate) to afford the desired compound as a crystalline solid (131 mg, 0.63 mmol, 88% yield); mp 138 °C; $[a]_D^{23}$ +85.0 (*c* 0.5, CH_3OH); ν_{max}(KBr)/cm^{-1} 3480 (NH), 1669 (C=O), 1636 (C=O); δ_H(CD_3OD, 500 MHz) 0.97 (3H, t, *J* 7.4, CH_3), 1.39–1.51 (2H, m, $CH_2CH_2CH_3$), 1.73–1.78 (2H, m, $CH_2CH_2CH_3$), 2.74 (1H, m, 7-H_a), 2.94 (1H, ddd, *J* 15.6, 7.3, 1.2, 7-H_b), 3.85 (1H, t, *J* 6.6, 3-H), 3.90 (1H, dq, *J* 16.0, 2.0, 9-H_a), 4.36 (1H, d, *J* 16.0, 9-H_b), 4.41 (1H, dd, *J* 10.8, 7.3, 6-H), 5.11 (1H, d, *J* 1.6, 8-C=CH_aH_b), 5.14 (1H, dd, *J* 2.0, 1.0, 8-C=CH_aH_b); δ_C(CD_3OD, 50 MHz) 13.9 (CH_3), 19.5 ($CH_2CH_2CH_3$), 36.7 and 37.4 ($CH_2CH_2CH_3$ and 7-CH_2), 50.9 (9-CH_2), 58.2 (3-CH), 59.1 (6-CH), 109.4 (8-C=CH_2), 142.3 (8-C), 168.4 (C=O), 170.8 (C=O); *m*/*z* (APCI$^+$, M + 1), 209 (100%); $C_{11}H_{17}N_2O_2$ requires 209.1290. Found 209.1295.

(3*S*,6*R*,8*R*)-3-Propyl-8-methyl-1,4-diazabicyclo[4.3.0]nonane-2,5-dione [*cyclo*-L-norvaline-(4*R*)-methyl-D-proline] 26

Palladium on carbon (20%, 15 mg) was added to a solution of DKP **38** (35 mg, 0.17 mmol) in methanol (5 ml) and stirred under an atmosphere of hydrogen overnight. The reaction mixture was filtered, and the solvent removed in vacuum to yield a crude gum [31 mg, 0.15 mmol, 89% yield, 85% de judged from integration of signals at δ 2.28 (minor) and δ 2.36 (major)]. The reaction mixture was purified by fractional recrystallisation (ethyl acetate) of the minor diastereoisomer **43** and concentration of the mother liquor to afford the desired compound **26** as a white crystalline solid (23 mg, 0.11 mmol, 65% yield); mp 231 °C (EtOAc); $[a]_D^{23}$ +168.0 (*c* 0.52, EtOH); δ_H($CDCl_3$, 500 MHz) 0.97 (3H, t, *J* 7.4, CH_3), 1.16 (3H, d, *J* 6.2, 8-CHCH_3), 1.41–1.56 (2H, m, $CH_2CH_2CH_3$), 1.68 (1H, q, *J* 10.8, 7-H_{endo}), 1.71–1.87 (2H, m, $CH_2CH_2CH_3$), 2.36 (1H, m, 8-H), 2.46 (1H, m, 7-H_{exo}), 3.18 (1H, dd, *J* 11.8, 9.7, 9-H_{endo}), 3.68 (1H, dd, *J* 11.8, 3.0, 9-H_{exo}), 3.92 (1H, td, *J* 7.4, 5.5, 3-H), 4.28 (1H, dd, *J* 10.8, 5.6, 6-H), 7.30 (1H, br d, *J* 4.8, NH); NOE enhancements ($CDCl_3$, 500 MHz) δ 1.16 (8-C-CH_3) enhances resonances at δ 1.68 (7-H_{endo}, 2.5%), δ 2.36 (8-H, 3.9%) and δ 3.18 (9-H_{endo}, 2.2%); δ 1.68 (7-H_{endo}) enhances resonances at δ 1.16 (8-C-CH_3, 3.8%); δ 2.36 (8-H, 2.2%), δ 2.46 (7-H_{exo}, 14.6%) and δ 3.18 (9-H_{endo}, 2.0%); δ 4.28 (6-H) enhances resonances at δ 2.36 (8-H, 4.0%) and δ 2.46 (7-H_{exo}, 5.8%); δ_C($CDCl_3$, 50 MHz) 13.5 (CH_3), 17.4 ($CH_2CH_2CH_3$), 18.6 (8-C-CH_3), 30.7, 36.1 and 37.1 ($CH_2CH_2CH_3$ and 7-CH_2 and 9-CH_2), 52.4 (8-CH), 57.7 and 58.7 (6-CH and 3-CH), 166.2 (C=O), 169.6 (C=O); *m*/*z* (APCI$^+$, M + 1); 211 (100%), $C_{11}H_{19}N_2O_2$ requires 211.1447. Found 211.1447.

***cyclo*-L-Ile-D-Pro 45**

Triethylamine (1.3 ml) was added to a solution of D-Pro-OMe·HCl (720 mg, 4.3 mmol) in $CHCl_3$ (20 ml) and the reaction mixture cooled to −78 °C. Isoleucine-*N*′-carboxyanhydride[41] (682 mg, 4.3 mmol) in THF (10 ml) was added dropwise and the reaction mixture stirred at −78 °C for 4 h. The reaction mixture was concentrated in vacuum to a volume of 10 ml, filtered through Celite with washing (ether), and the solvent removed in vacuum to afford a crude oil. This oil was dissolved in ethanol (20 ml), heated at reflux for 36 h, the solvent removed in vacuum, and the crude oil purified by chromatography (ethyl acetate) to afford the desired compound as an amorphous white powder (611 mg, 2.9 mmol, 67%); $[a]_D^{23}$ +98 (*c* 0.1, EtOH); δ_H($CDCl_3$, 500 MHz) 0.95 (3H, t, *J* 7.4, CH_3), 1.04 (3H, d, *J* 6.9, CH_3), 1.21–1.31 (1H), 1.55–1.62 (1H, m), 1.86–2.08 (4H, m), 2.41–2.45 (1H, m), 3.52 (1H, m), 3.74 (1H, m), 3.81 (1H, m), 4.10 (1H, m), 6.37 (1H, br, NH); δ_C($CDCl_3$, 50 MHz) 11.3, 15.3, 22.0, 24.5, 29.4, 39.7, 45.6, 58.3, 62.9, 165.2, 169.2; *m*/*z* (APCI$^+$, M + 1), 211 (100%); $C_{11}H_{18}N_2O_2$ requires 211.1447. Found 211.1443.

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