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Enzymatic resolution of (±)-5-phenyl-4,5-dihydroisoxazole-3-carboxylic acid ethyl ester and its transformations into polyfunctionalised amino acids and dipeptides

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ABSTRACT

Enantiomerically pure (5*R*)-(−)-5-phenyl-4,5-dihydroisoxazole-3-carboxylic acid ethyl ester was obtained via enzymatic resolution of the corresponding racemic mixture using a lipase from hog pancreas (PPL). The following reduction of the ester group to the corresponding alcohol and the oxidation of the latter led to (5*R*)-(−)-5-phenyl-4,5-dihydroisoxazole-3-carbaldehyde, and the reaction between this and *Schöllkopf*'s reagent, (2*R*)-(+)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine, gave mixtures of adducts with a good *syn*/*anti* ratio. The steric configurations of the major diastereoisomer were assigned on the basis of spectroscopic data and X-ray analysis. The subsequent controlled hydrolysis of the pyrazine ring led to β-(5-phenyl-4,5-dihydroisoxazol-3-yl)-serine methyl esters and the corresponding dipeptides with (*R*)-valine. Finally, reductive cleavage of the 4,5-dihydroisoxazole ring under hydrolytic conditions made it possible to obtain the corresponding polyfunctionalised dipeptides.

1. Introduction

Many biologically active natural products, such as cyclomarins,[1a] polyoxins[1b] and vancomycin[1c] include β-hydroxy-α-amino acids within their structure. A number of studies have investigated the stereoselective synthesis of this unit[2] using asymmetric aldol reactions with chiral auxiliaries[3] or chiral catalysts.[4] Among the various chiral glycine equivalents,[5] *Schöllkopf*'s bislactim ether (i.e., (2*R*)- or (2*S*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine)[6] is a particularly attractive chiral auxiliary because of its high degree of selectivity in aldol-type reactions, in addition to the fact that enantiopure (*R*)- and (*S*)-forms are both commercially available.

Our interest in the stereoselective synthesis of new non-proteinogenic β-hydroxy-α-amino acids substituted with heterocyclic rings by means of the reaction between *Schöllkopf*'s reagent and heterocyclic-carbaldehydes[7] has recently led us to consider the 4,5-dihydroisoxazole (2-isoxazoline) as a selected heterocycle,[8] because it is easily prepared, versatile as a synthetic intermediate in a wide variety of complex natural products[9] and structurally relevant to medicinal chemistry.[10] As synthons, 4,5-dihydroisoxazoles can be converted into a number of useful synthetic units, such as β-hydroxy ketones[11] and γ-amino alcohols,[12] depending on the experimental conditions used for the reductive ring cleavage. With the aim of obtaining new polyfunctionalised-α-amino acids with a supplementary homochiral stereocentre after cleavage of the dihydropyrazine and isoxazoline rings, we decided to extend the protocol used with 5,5-disubstituted-4,5-dihydroisoxazole-3-carbaldehydes[8] to the reaction between *Schöllkopf*'s reagent (2*R*)-**1** and (5*R*)-5-phenyl-4,5-dihydroisoxazole-3-carbaldehyde **2**. The 5-aryl-4,5-dihydroisoxazole nucleus is of particular interest because it is present in several compounds used as antiparasitics, pesticides, insecticides and fungicidal agents.[13] Aldehyde **2** was already known but only in racemic form, and so we also investigated different ways of obtaining enantiomerically pure aldehyde **2** with the aim of minimising the total number of diastereoisomers derived from the reaction with *Schöllkopf*'s reagent.

2. Results and discussion

Following a recently described method,[14] we prepared the racemic 5-phenyl-4,5-dihydroisoxazole-3-carboxylic acid ethyl ester (±)-**3** by means of a base-catalysed condensation between ethyl nitroacetate and styrene (Scheme 1).

Various routes were attempted to resolve the racemic mixture of ester **3** (Scheme 2). First, it was hydrolysed to the corresponding carboxylic acid[15] **4** and this was treated with either (*R*)- or (*S*)-1-phenyl-ethylamine with the aim of obtaining a mixture of

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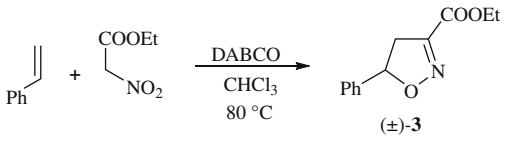


Scheme 1.

diastereoisomeric salts separable by fractional crystallisation but, unfortunately, this was not possible even when using different crystallisation solvents.[16]

Ester **3** was then transformed into a couple of diastereoisomeric esters **5a,b** by means of *trans*-esterification with different chiral alcohols such as L-menthol and (*S*)-2-methyl-1-butanol but in this case, it was not possible to separate the diastereoisomeric-obtained mixtures chromatographically. The same problem was encountered when transforming acid **4**, after activation by the *Mukaiyama's* reagent, into the pair of diastereoisomeric amides **6** by means of a reaction with (*S*)-methyl-(1-phenyl-ethyl)-amine.

Having failed with these approaches to separate the enantiomers, we turned our attention to an enzymatic resolution.

To obtain the enantiomerically pure aldehyde **2**, it is possible to use two different biocatalytical approaches based on kinetic resolution: the reduction of aldehyde (±)-**2** catalysed by yeasts or the hydrolysis of ester (±)-**3** by the same microorganisms or isolated hydrolases.

In the case of aldehyde reduction, the yeasts which were used showed good activity although alcohol **7** was obtained as a racemic mixture.

The hydrolysis of ester (±)-**3** was preliminarily screened using different genera of non-conventional yeasts and commercial lipase and acylase; all of the catalysts hydrolysed the substrate with a good rate, but only PPL and *Pichia etchellsii* MIM selectively hydrolysed the ester function of **3**. Evaluation of the progress of the reaction showed that the enantiomerically pure ester could only be obtained by driving the reaction to over 50% of molar conversion (Table 1).

The best results were obtained using PPL, which was also most active at a low concentration (5 g L^{-1}). In this case it was possible to obtain the enriched unreacted ester (−)**3** with 65% of molar conversion and 96% ee (Scheme 3).

The absolute configuration of ester (−)-**3** was not assigned at this stage, but was determined by means of X-ray analysis of the major adduct obtained in the next reaction with *Schöllkopf*'s reagent (see below) and proved to be (5*R*). Ester (−)-**3** was then reduced by sodium borohydride[17] into the alcohol (−)-**7**, and oxidation of the latter with manganese dioxide led to (5*R*)-5-phenyl-4,5-dihydroisoxazole-3-carbaldehyde (−)-**2** (Scheme 4).

Table 1
Hydrolysis of ester (±)-**3** with resuspended microbial cells and PPL

Biocatalysts	ee **3**	ee **4**	Molar conversion[a] (%)	*E*[a]	Time
PPL	60	75	44	12	30 min
PPL	96	52	65	11	45 min
Pichia etchellsii MIM	44	47	48	4.2	2 h
Pichia etchellsii MIM	70	45	61	5.3	3.5 h

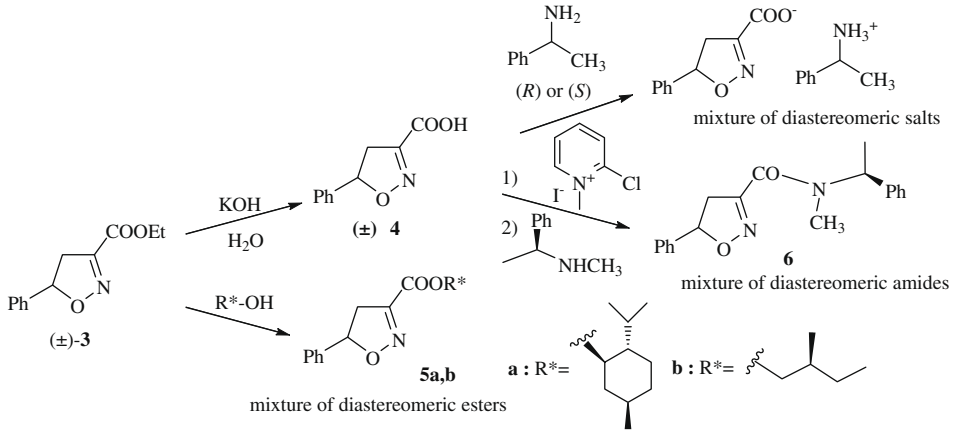
[a] Conversion and enantioselectivity factor (*E*) were calculated from the ee of the substrate and the product.

In accordance with a general procedure, a solution of aldehyde (−)-**2** was added to the anion of the bislactim ether (*R*)-**1** generated by *n*BuLi in THF at $T = -78$ °C. To evaluate the influence of the counter-ion on diastereoselectivity, the reaction was also performed in a parallel experiment in which the lithium azaenolate was treated with triisopropoxytitanium(IV) chloride[18] to give the corresponding titanium salt before the addition of aldehyde **2**. Both reaction mixtures were maintained at the same temperature for 6 h before being treated with an aqueous phosphate buffer solution. TLC analysis and the ^{1}H NMR spectrum of the crude reactions made it possible to establish the presence of a mixture of diastereoisomers **8**–**11**, whose precise ratio was determined by means of HPLC analysis (Scheme 5 and Table 2).

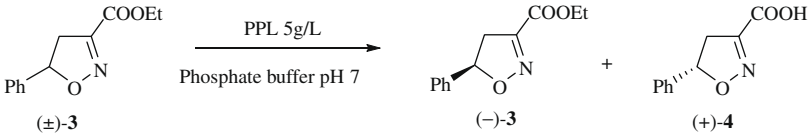
When the reaction temperature was increased to −20 °C, the yield of the adducts decreased, and the (*R*)-3-hydroxy-3-phenylpropionitrile[19] was isolated as a by-product (20%) (Fig. 1). Similarly to the reported decarboxylative ring-opening reaction of 3-carboxyisoxazolines,[20] we hypothesised that the anion of the alcohol evolves and a side ring-opening process involving the isoxazoline ring takes place as shown in Figure 1.

Diastereoisomers **8**–**11** were purified by flash chromatography on silica gel, and their structures were confirmed on the basis of analytical and spectroscopic data. The (2*S*)-configuration of compounds **8** and **10** was established using the $^5J_{2H/5H}$ coupling constant value of approximately 3.6 Hz, which corresponds to a *trans*-relationship between the 2-H and 5-H protons of the pyrazine ring.[21] The major adduct **8** was obtained as a crystalline solid from a solution of CH_2Cl_2/^{i}Pr$_2$O and underwent X-ray crystallographic analysis (Fig. 2).

This allowed us to assign the (*R*)-configuration to the 5-C of the isoxazoline ring (also in compounds **3**, **7** and **2**) and the (*S*)-configuration to both 1′-C and pyrazine-2-C. As a consequence, the (*R*)-configuration was assigned to the 1′-C of epimer **10**.



Scheme 2.



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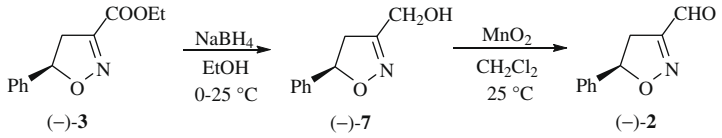
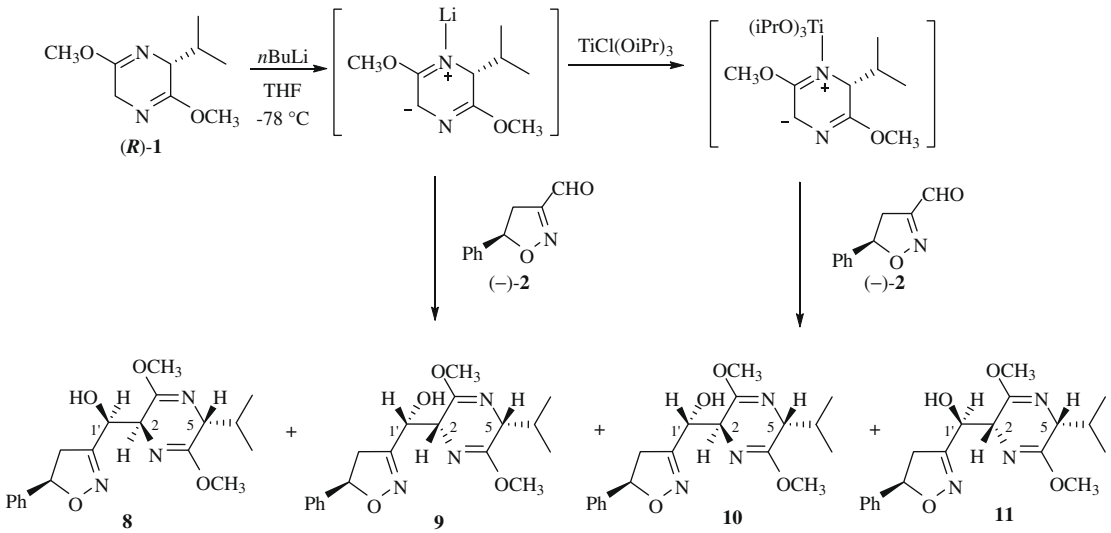


Scheme 4.



Scheme 5.

Table 2

Counterion	Total yield (%)	Diastereomer ratios					
		8 (2*S*,1′*S*)	**9** (2*R*,1′*R*)	**10** (2*S*,1′*R*)	**11** (2*R*,1′*S*)	**8+10/ 9+11**	**8/10**
Li	60	56.8	21.9	19.5	1.8	3/1	3/1
Ti	60	76.8	4.5	18.7	0.0	21/1	4/1

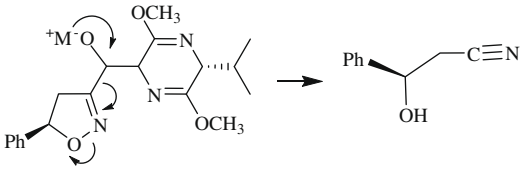


Figure 1.

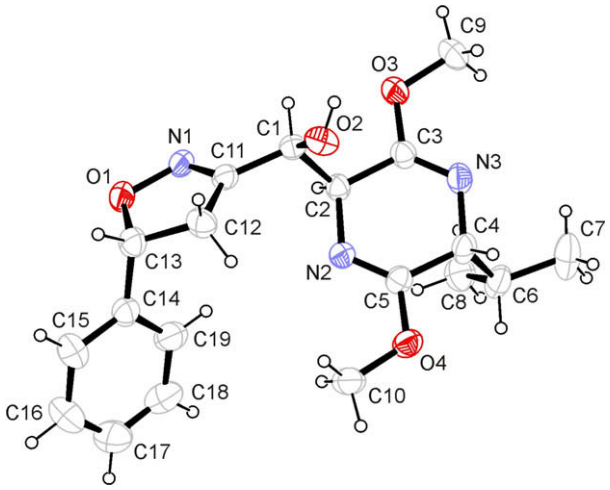


Figure 2. ORTEP plot of **8** with atom numbering scheme. Displacement ellipsoids at 30% probability level.

On the contrary, the ^{1}H NMR spectra of diastereoisomers **9** and **11** showed a $^{5}J_{2H/5H}$ coupling constant value of approximately 5.6 Hz, which corresponds to a *cis* relationship between the 2-H and 5-H protons of the pyrazine ring, and was confirmed by a positive NOE between the two protons. The (1′*R*) and (1′*S*) configurations were, respectively, assigned to diastereoisomers **9** and **11** taking into account the accepted model for the aldol-type addition of **1** to aldehydes,[6b] which has also been extensively confirmed in our previous studies.[7e,8] On the strength of this model, the aldehyde attacks the azaenolate-pyrazine by means of a more favourable

transition state in which the aldehyde substituent is far from the methoxy group and the metal atom, with the consequent predominance of the adduct (1′*S*)-**8** when the attack takes place from the opposite side of the isopropyl group. On the contrary, when the attack takes place from the same side as the isopropyl group, the most favourable transition state is that leading to compound (1′*R*)-**9** (Fig. 3).

This is the first time we have observed the formation of products arising from an attack of the aldehyde from the more hindered side of the azaenolate (adducts **9** and **11**),[22,23] but the formation of the more abundant diastereoisomers obtained using the lithium counterion (adducts **8** and **9**) is in agreement with *Schöllkopf*'s model. Because of the well known tight transition state generally promoted by the titanium ligand,[21a] the reaction with the titanium azaenolate proceeded more selectively than that with the lithium salt. As shown in Table 2, the diastereofacial selectivity with respect to the pyrazine anion is enhanced (Σ(2*S*):Σ(2*R*) = 21:1 vs 3:1) as is the facial preference of the carbonyl addition, albeit in a less marked manner (ratios (1′*S*):(1′*R*) = 4:1 vs 3:1).

An involvement of the isoxazolidine ring in the complex intermediate can be expected especially when $TiCl(OiPr)_3$ is used.[24] However, in this case, the additional coordination of the titanium atom with the isoxazoline nitrogen should involve a less stable *s-cis* O=C–C=N conformation of the aldehyde, as well as a more encumbered transition state with the isoxazoline arrangement on the same side as the methoxy group. In fact, the quantity of the derived adduct **10** was practically the same with the two counterions (Table 2), thus indicating that the metal-isoxazolidine coordination had no significant effect.

Finally, the presence of the (*R*)-stereocentre at the 5-position of the isoxazoline ring may promote the formation of adduct **9** because it causes less steric hindrance in the corresponding transition state, especially in the looser transition state with the lithium counterion.

Adducts **8** and **9** were hydrolysed under controlled conditions: they were treated with 3 equiv of 0.2 M HCl in THF at room temperature for 24 h, which allowed the isolation of the β-substituted serine methyl esters **12**, **13** and the dipeptides **14**, **15** (Scheme 6).

Given the partial hydrolysis of the pyrazine ring, the dipeptide formation was observed early in the hydrolysis reactions.[8,25] Products **12–13** and **14–15** were separated by means of column chromatography and their structure was assigned using ^1H NMR analysis.[8,25b,c]

Finally, the hydrogenolysis–hydrolysis of the 4,5-dihydroisoxazole ring of dipeptide **13** using 3 equiv of $B(OH)_3$ in a mixture $MeOH/H_2O$, with H_2 and Raney-Ni as catalyst,[11b] led to the corresponding β,ε-dihydroxy-γ-oxo α-amino acid derivative **16** in good yield (Scheme 6). This α-amino acid derivative, like the ε,ε-disubstituted derivatives we had previously obtained, has a highly functionalised structure with a further stereocentre that makes it extremely attractive as a potential peptidomimetic.

3. Conclusion

(5*R*)-(−)-5-Phenyl-4,5-dihydroisoxazole-3-carboxylic acid ethyl ester was obtained by means of enzymatic resolution using a lipase from hog pancreas. *Schöllkopf*'s reagent was used as a chiral auxiliary to introduce an amino acid residue in the subsequent reaction with the corresponding aldehyde. The hydrolysis and hydrogenolysis of adducts **8** and **9** allowed us to obtain β,ε-dihydroxy-γ-oxo α-amino acid derivatives. These compounds may be interesting because 2-isoxazoline derivatives have been used as dipeptide bioisosteres[26] and incorporated into biologically active compounds such as the anti-tumour drug acivicin.[27]

4. Experimental

4.1. General methods

Melting points were measured using a *Büchi* apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ using a *Bruker AC 300* spectrometer; the chemical shifts (δ) are given in ppm relative to TMS, and all of the coupling constants are in Hertz. The optical rotation values were measured at 25 °C using a *JASCO P-1030* spectropolarimeter. The MS spectra were determined using a *VG Analytical 7070 EQ* mass spectrometer with an attached *VG analytical 11/250* data system. The IR spectra (in cm^{-1}) were determined using a *Jasco FT-IR 4100* spectrometer.

(2*R*)-2,5-Dihydro-3,6-dimethoxy-2-isopropylpyrazine **1** and ethyl nitroacetate were obtained from commercial sources.

4.2. General procedure for the preparation of compounds 5a,b

A mixture of ester **3** (2 mmol, 1 equiv), chiral alcohol [L-menthol or (*S*)-2-methyl-1-butanol] (2 mmol, 2 equiv) and *t*BuOK (0.4 mmol, 0.2 equiv) in toluene (15 mL) was heated at 110 °C for three days.

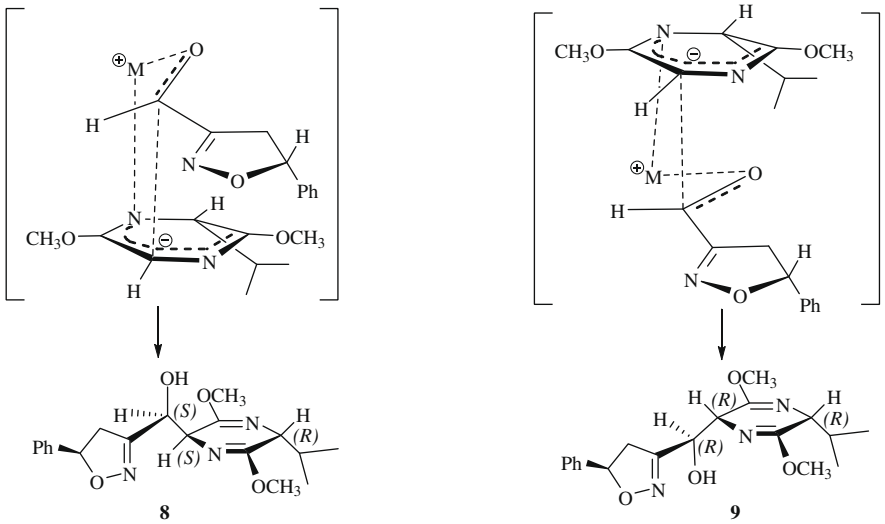
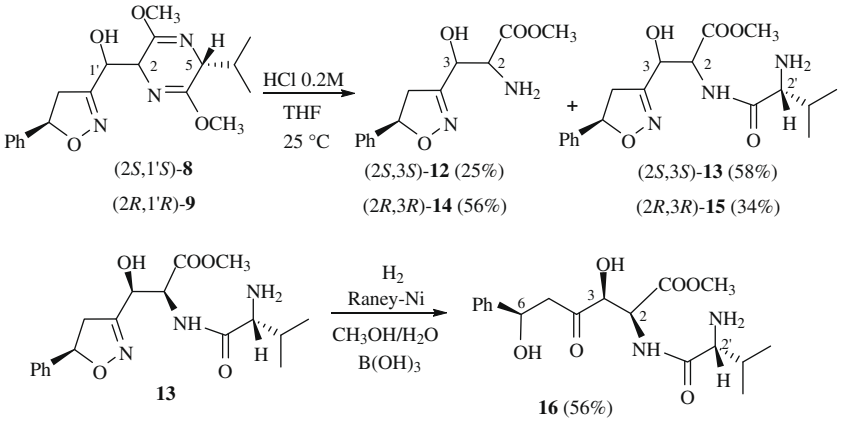


Figure 3. Transition states leading to the major diastereoisomeric adducts.



Scheme 6.

The organic solvent was evaporated off, and the residue was treated with water and extracted with several portions of ethyl acetate. The combined extracts were dried (Na_2SO_4) and concentrated at reduced pressure. The crude esters were purified by column chromatography on silica gel (hexane/ethyl acetate = 8/2).

4.3. 5-Phenyl-4,5-dihydroisoxazole-3-carboxylic acid (2*S*)-isopropyl-(5*R*)-methyl-(1*R*)-cyclohexyl ester 5a (mixture of diastereoisomers)

Oil (40%); ^{1}H NMR: δ 0.80–2.10 (18H, m, cyclohex.); 3.10–3.20 (1H, 2dd, 4-H isox.); 3.60–3.70 (1H, 2dd, 4-H isox.); 4.82 (1H, dt, J = 4.4, 10.9, 1-H cyclohex.); 5.75–5.90 (1H, dd, J = 9.4, 11.4, 5-H isox.); 7.38–7.50 (5H, m, Ph). MS-EI (m/z): 329 (M^+).

4.4. 5-Phenyl-4,5-dihydroisoxazole-3-carboxylic acid (2*S*)-methyl-butyl ester 5b (mixture of diastereoisomers)

Oil (35%); ^{1}H NMR: δ 0.93 (3H, t, J = 7.4, 4-CH_3 butyl); 0.97 (3H, d, J = 6.7, 2-CH_3 butyl); 1.25 (1H, m, 3-H butyl); 1.50 (1H, m, 3-H butyl); 1.85 (1H, m, 2-H butyl); 3.20 (1H, dd, J = 9.0, 17.8, 4-H isox.); 3.65 (1H, dd, J = 11.6, 17.8, 4-H isox.); 4.15 (2H, m, 1-H butyl); 5.82 (1H, dd, J = 9.0, 11.6, 5-H isox.); 7.30–7.50 (5H, m, Ph). MS-EI (m/z): 261 (M^+).

4.5. 5-Phenyl-4,5-dihydroisoxazole-3-carboxylic acid methyl-[(1*S*)-phenyl-ethyl]-amide 6 (mixture of diastereoisomers)

To a solution of acid **4** (0.38 g, 2 mmol, 1 equiv) in CH_2Cl_2 (15 mL), TEA (0.29 mL, 2.2 mmol) and *Mukaiyama's* reagent (0.51 g, 2 mmol, 1 equiv) were added. The mixture was heated at reflux temperature for 4 h. Then (*S*)-methyl-(1-phenyl-ethyl)-amine (0.29 mL, 2 mmol, 1 equiv) was added and the heating was continued for 11 h. The mixture was cooled to room temperature and washed with water, and then with 10% HCl and 10% $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed (silica gel, ethyl acetate/CH_3OH = 95/5) to afford a colourless oil (72%). ^{1}H NMR: δ 1.55–1.70 (3H, m, CH_3 ethyl); 2.76 (3H, d, J = 4.1, N–CH_3 1° diast); 2.94 (3H, s, N–CH_3 2° diast); 3.30–3.52 (1H, m, 4-H isox.); 3.70–3.85 (1H, m, 4-H isox.); 5.70 (1H, m, Ph–CH–N); 6.00 (1H, m, 5-H isox. 1° diast); 6.15 (1H, m, 5-H isox. 2° diast); 7.44 (10H, m, Ph). MS-EI (m/z): 308 (M^+).

4.6. Biotransformation conditions and analytical method

Aldehyde (±)-**2** was reduced and ester (±)-**3** was hydrolysed in 10 mL screw-capped test tubes with a reaction volume of 3 mL using different yeasts (20 g L^{-1}, dry weight) grown on a malt extract with 0.5% of yeast extract for 48 h at 28 °C on a reciprocal shaker. The yeasts were resuspended in 0.1 M phosphate buffer, pH 7, with 5% glucose added in the case of the reduction. The substrate was dissolved in DMSO and added to the biotransformation system to give 4 mg L^{-1} of substrate concentration and 2% of solvent. The reactions were carried out at 28 °C under magnetic stirring. The hydrolysis of ester (±)-**3** was also carried out using 5 g L^{-1} of different commercial lipases and acylases.

The enantiomeric excess and molar conversion were determined by means of chiral HPLC analysis using a Chiralpak AD analytical column and a mixture of hexane/*iso*PrOH:90/10 with a flow rate of 0.6 ml/min. The samples (0.5 ml) were extracted with ethyl acetate, and the aqueous phase was brought to pH 2 with 5% HCl and twice extracted with an equal volume of ethyl acetate. The organic phases were dried over Na_2SO_4, after which the solvent was removed and the acid was methylated with (trimethylsilyl)-diazomethane.

4.7. (5*R*)-5-Phenyl-4,5-dihydroisoxazole-3-carboxylic acid ethyl ester 3

Compound (−)-**3** was obtained by biotransformation, with 3.57 g of ester (±)-**3** dissolved in DMSO and 3.75 g of Lipase from hog pancreas (PPL) being added to 700 ml of 0.1 M phosphate buffer, pH 7. The biotransformation was carried out at 30 °C under magnetic stirring. After 45 min (HPLC monitoring), the reaction was extracted three times with ethyl acetate to recover ester (−)-**3**. The aqueous phase was brought to pH 2 with HCl and extracted three times with ethyl acetate to recover the acid (+)-**4**. The organic extracts were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude **3** was purified by means of flash chromatography on silica gel (hexane/ethyl acetate = 90/10). Colourless oil (30%); $[\alpha]_D^{20} = -285.3$ (c 0.95, $CHCl_3$). The spectroscopic data are in agreement with those reported for the racemic compound.[14]

4.8. [(5*R*)-5-Phenyl-4,5-dihydroisoxazol-3-yl]-methanol 7

Alcohol (−)-**7** was prepared starting from the ester (−)-**3** as previously described for the racemic compound.[17] Colourless solid (94%); mp 68–70 °C (hexane–cyclohexane); $[\alpha]_D^{20} = -166.3$ (c 1.05, $CHCl_3$). ^{1}H NMR: δ 1.60 (1H, broad s, OH); 3.10 (1H, dd, J = 8.4, 17.1, 4-H); 3.55 (1H, dd, J = 10.8, 17.1, 4-H); 4.48 (2H, s, CH_2O); 5.65 (1H, dd, J = 8.4, 10.8, 5-H); 7.45 (5H, m, Ph).

4.9. (5*R*)-5-Phenyl-4,5-dihydroisoxazole-3-carbaldehyde 2

Aldehyde (−)-**2** was prepared starting from the alcohol (−)-**7** with MnO_2.[8] Colourless oil[17] (83%); $[\alpha]_D^{20} = -459$ (c 1.29, $CHCl_3$).

^{1}H NMR: δ 3.16 (1H, dd, J = 8.9, 17.7, 4-H); 3.58 (1H, dd, J = 11.6, 17.7, 4-H); 5.86 (1H, dd, J = 8.9, 11.6, 5-H); 7.30–7.50 (5H, m, Ph); 10.03 (1H, s, CHO).

4.10. Reaction of (*R*)-1 with (−)-2

Butyl lithium (0.81 mL of a 1.6 M solution in hexane, 1.3 mmol, 1.05 equiv) was added to a solution of (*R*)-**1** (0.22 mL, 1.23 mmol, 1 equiv) in anhydrous THF (5 mL) cooled at −78 °C, and the mixture was stirred for 45 min. A solution of triisopropoxytitanium(IV) chloride[18] (1.33 mmol, 1.075 equiv), prepared by mixing titanium tetraisopropoxide (1.0 mmol, 0.3 mL) in anhydrous hexane (2 mL) and titanium tetrachloride (0.33 mmol, 0.32 mL of a 1 M solution in toluene), was added and stirring was continued for a further 45 min. Aldehyde (−)-**2** (0.216 g, 1.23 mmol, 1 equiv) in THF (4 mL) was added, and the mixture was stirred at −78 °C for 6 h. The reaction mixture was allowed to warm to −10 °C, after which a phosphate buffer solution (10 mL) having a pH of 7 was added, and the mixture was extracted with CH_2Cl_2. The organic phase was separated and dried with Na_2SO_4, and the solvent was evaporated in vacuo. Compounds **8–11** were purified by means of flash chromatography on silica gel (hexane/ethyl acetate = 80/20) and subsequently separated by means of flash chromatography on silica gel (Supelco, Versaflash® station, CH_2Cl_2/ethyl acetate = 95/5). The diastereoisomeric ratio of compounds **8**–**10** was determined by means of HPLC analysis (Agilent 1100 Series equipped with detector diode array on Supelco Ascentis® Si column, hexane/*iso*-PrOH=95/5, flow: 0.7 mL min^{-1}).

4.11. (*S*)-[(2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl]-[(5*R*)-5-phenyl-4,5-dihydroisoxazol-3-yl]-methanol 8

Colourless solid; mp 92–94 °C (*iso*Pr_2O); $[\alpha]_D^{20} = -149.35$ (c 0.96, $CHCl_3$). ^{1}H NMR: δ 0.75, 1.08 (6H, 2d, J = 6.8, CH(CH_3)$_2$); 2.29 (1H, m, CH(CH$_3$)$_2$); 2.84 (1H, d, J = 7.5, OH); 3.19 (1H, dd, J = 17.0, 8.0, 4-H); 3.59 (1H, dd, J = 17.0, 10.9, 4-H); 3.66 (3H, s, OCH$_3$); 3.77 (3H, s, OCH$_3$); 4.04 (1H, t, J = 3.6, 5-H pyraz.); 4.26 (1H, t, J = 3.6, 2-H); 5.00 (1H, dd, J = 7.5, 3.6, 1′-H); 5.67 (1H, dd, J = 10.9, 8.0, 5-H isox.); 7.34–7.40 (5H, m, Ph); (by deuteration the signal at 2.84 disappeared and the signal at 5.00 turned into a doublet with J = 3.6). ^{13}C NMR: δ 16.78, 19.00 (CH(*C*H$_3$)$_2$); 31.94 (*C*H(CH$_3$)$_2$); 43.26 (4-C); 52.78 (3- and 6-OCH$_3$); 59.33, 61.02 (2-C and 5-C pyr.); 68.90 (1′-C); 82.22 (5-C isox.); 125.67, 128.09, 128.62, 141.05 (Ph); 159.52, 160.33, 166.60 (3-C and 6-C pyr., 3-C isox.). MS-FAB$^+$ (m/z): 360 (MH$^+$). Anal. Calcd for $C_{19}H_{25}N_3O_4$: C, 63.51; H, 6.96; N, 11.69. Found: C, 63.21; H 6.74; N, 11.49. IR (nujol): 3378 (ν_{OH}, OH), 1646 ($\nu_{C=N}$, C=N). Single crystals suitable for X-ray structure determination were obtained by precipitation from CH_2Cl_2/*iso*Pr_2O = 1/1. HPLC analysis: retention time = 11.2 min.

4.12. (*R*)-[(2*R*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl]-[(5*R*)-5-phenyl-4,5-dihydroisoxazol-3-yl]-methanol 9

Oil; $[\alpha]_D^{20} = -78.7$ (c 1.41, $CHCl_3$). ^{1}H NMR: δ 0.79, 1.13 (6H, 2d, J = 6.8, CH(CH_3)$_2$); 2.37 (1H, m, CH(CH$_3$)$_2$); 2.79 (1H, d, J = 6.8, OH); 3.20 (1H, dd, J = 16.9, 8.1, 4-H); 3.59 (1H, dd, J = 16.9, 11.0, 4-H); 3.69 (3H, s, OCH$_3$); 3.78 (3H, s, OCH$_3$); 4.00 (1H, dd, J = 5.6, 3.7, 5-H pyraz.); 4.25 (1H, dd, J = 5.6, 4.0, 2-H); 4.97 (1H, dd, J = 6.8, 4.0, 1′-H); 5.70 (1H, dd, J = 11.0, 8.1, 5-H isox.); 7.34–7.45 (5H, m, Ph); (by deuteration the signal at 2.79 disappeared and the signal at 4.97 turned into a doublet with J = 4.0). ^{13}C NMR: δ 17.03, 19.48 (CH(*C*H$_3$)$_2$); 30.95 (*C*H(CH$_3$)$_2$); 42.75 (4-C); 52.49,52.58 (3- and 6-OCH$_3$); 58.71, 60.60 (2-C and 5-C pyr.); 68.92 (1′-C); 82.45 (5-C isox.); 125.79, 128.06, 128.51, 140.96 (Ph); 159.1, 159.18, 165.79 (3-C and 6-C pyr., 3-C isox.). MS-FAB$^+$ (m/z): 360 (MH$^+$). Anal. Calcd for $C_{19}H_{25}N_3O_4$: C, 63.51; H, 6.96; N, 11.69. Found: C, 63.37; H 6.69; N, 11.54. IR (nujol): 3448 (ν_{OH}, OH), 1696 ($\nu_{C=N}$, C=N). HPLC analysis: retention time = 5.8 min.

4.13. (*R*)-[(2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl]-[(5*R*)-5-phenyl-4,5-dihydroisoxazol-3-yl]-methanol 10

Amorphous solid; $[\alpha]_D^{20} = -51.2$ (c 0.65, $CHCl_3$). ^{1}H NMR: δ 0.74, 1.05 (6H, 2d, J = 6.8, CH(CH_3)$_2$); 2.28 (1H, m, CH(CH$_3$)$_2$); 2.93 (1H, dd, J = 17.0, 7.5, 4-H); 3.33 (1H, dd, J = 17.0, 10.9, 4-H); 3.59 (1H, d, J = 7.9, OH); 3.73 (3H, s, OCH$_3$); 3.76 (3H, s, OCH$_3$); 3.90 (1H, t, J = 3.5, 5-H pyraz.); 4.36 (1H, broad t, J = 4.1, 2-H); 4.95 (1H, dd, J = 7.9, 4.6, 1′-H); 5.58 (1H, dd, J = 10.9, 7.5, 5-H isox.); 7.30–7.45 (5H, m, Ph); (by deuteration the signal at 3.59 disappeared and the signal at 4.95 turned into a doublet with J = 4.6). ^{13}C NMR: δ 16.75, 18.92 (CH(*C*H$_3$)$_2$); 32.05 (*C*H(CH$_3$)$_2$); 43.32 (4-C); 52.60, 52.84 (3- and 6-OCH$_3$); 58.68, 61.26 (2-C and 5-C pyr.); 69.07 (1′-C); 81.92 (5-C isox.); 125.67, 128.10, 128.68, 140.99 (Ph); 157.71, 160.22, 165.73 (3-C and 6-C pyr., 3-C isox.). MS-FAB$^+$ (m/z): 360 (MH$^+$). IR (nujol): 3432 (ν_{OH}, OH), 1642 ($\nu_{C=N}$, C=N). HPLC analysis: retention time = 14.8 min.

4.14. (*S*)-[(2*R*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl]-[(5*R*)-5-phenyl-4,5-dihydroisoxazol-3-yl]-methanol 11

This adduct was obtained only in the reaction with a Li counterion. Amorphous solid; $[\alpha]_D^{20} = -41.4$ (c 0.45, $CHCl_3$). ^{1}H NMR: δ 0.68, 1.08 (6H, 2d, J = 6.8, CH(CH_3)$_2$); 2.30 (1H, m, CH(CH$_3$)$_2$); 3.03 (1H, dd, J = 16.8, 8.6, 4-H); 3.50 (1H, dd, J = 16.8, 11.2, 4-H); 3.54 (1H, broad, OH); 3.59 (3H, s, OCH$_3$); 3.72 (3H, s, OCH$_3$); 3.93 (1H, dd, J = 5.5,3.5, 5-H pyraz.); 4.22 (1H, broad t, J = 6.2, 2-H); 4.72 (1H, broad t, J = 5.7, 1′-H); 5.57 (1H, dd, J = 11.2, 8.6, 5-H isox.); 7.23–7.40 (5H, m, Ph); (by deuteration the signal at 3.54 disappeared and the signal at 4.72 turned into a doublet with J = 6..6). ^{13}C NMR: δ 17.12, 19.64 (CH(*C*H$_3$)$_2$); 30.78 (*C*H(CH$_3$)$_2$); 42.44 (4-C); 52.75, 52.95 (3- and 6-OCH$_3$); 58.52, 60.87 (2-C and 5-C pyr.); 70.39 (1′-C); 82.06 (5-C isox.); 126.02, 128.10, 128.64, 141.26 (Ph); 158.16, 160.18, 164.99 (3-C and 6-C pyr., 3-C isox.). MS-FAB$^+$ (m/z): 360 (MH$^+$). IR (nujol): 3432 (ν_{OH}, OH), 1640 ($\nu_{C=N}$, C=N). HPLC analysis: retention time = 10.7 min.

4.15. General procedure for the hydrolysis of adducts 8 and 9

Adducts **8** and **9** (0.5 mmol) were dissolved in THF (7.5 mL) and a 0.2 M solution of HCl (7.5 mL, 1.5 mmol, 3 equiv) was added. The mixture was stirred for 24 h at room temperature, and then extracted with diethyl ether in order to remove non-basic organic compounds. It was then treated with 25% ammonia solution under stirring until pH 8–10, and extracted with CH_2Cl_2 (5 × 20 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was removed in vacuo. Compounds **12**, **13** and **14**, **15** were separated by means of flash chromatography (SiO_2, ethyl acetate/methanol = 98:2, developer: I_2).

4.16. (2*S*)-Amino-(3*S*)-hydroxy-3-[(5*R*)-phenyl-4,5-dihydroisoxazol-3-yl]-propionic acid methyl ester 12

Oil (25%); R_f = 0.27 (ethyl acetate/methanol = 95:5); $[\alpha]_D^{20} = -54.1$ (c 0.77, $CHCl_3$). ^{1}H NMR: δ 2.30–2.80 (3H, broad, OH, NH$_2$); 3.16 (1H, dd, J = 17.1, 7.9, 4-H isox.); 3.47 (1H, dd, J = 17.1, 11.2, 4-H isox.); 3.76 (3H, s, OCH$_3$); 3.98 (1H, m, 2-H); 4.72 (1H, m, 3-H); 5.62 (1H, dd, J = 11.2, 7.9, 5-H isox.); 7.25–7.45 (5H, m, Ph). ^{13}C NMR: δ 42.42 (4-C-isox.); 52.68 (OCH$_3$); 56.28 (2-C); 68.42 (3-C); 82.52 (5-C-isox.); 125.90, 128.26, 128.73, 140.55 (Ph); 157.16, 174.35 (C=N, C=O). MS-FAB$^+$ (m/z): 265 (MH$^+$). IR (nujol): 3374 (ν_{OH}, ν_{NH}, OH, NH$_2$), 1741 ($\nu_{C=O}$, C=O), 1677 ($\nu_{C=N}$, C=N).

4.17. (2*S*)-[(2*R*)-Amino-3-methyl-butyrylamino]-(3*S*)-hydroxy-3-[(5*R*)-5-phenyl-4,5-dihydroisoxazol-3-yl]-propionic acid methyl ester 13

Amorphous solid (58%); R_f = 0.16 (ethyl acetate/methanol = 95:5); $[\alpha]_D^{20} = -80.7$ (*c* 0.32, $CHCl_3$). ^{1}H NMR: δ 0.86, 0.99 (6H, 2d, *J* = 6.8, CH(CH_3)$_2$); 2.24 (1H, m, $CH(CH_3)_2$); 2.51–2.70 (3H, broad, OH, NH_2); 2.96 (1H, dd, *J* = 17.0, 9.0, 4-H isox.); 3.34 (1H, m, 2′-H); 3.62 (1H, dd, *J* = 17.0, 10.7, 4-H isox.); 3.77 (3H, s, OCH_3); 4.94 (1H, dd, *J* = 8.4, 2.3, 2-H); 5.00 (1H, broad d, *J* = 2.3, 3-H); 5.60 (1H, dd, *J* = 10.7, 9.0, 5-H isox.); 7.25–7.50 (5H, m, Ph); 8.21 (1H, d, *J* = 8.4, NH–CO); (by deuteration the signals at 2.51–2.7 and 8.21 disappeared and the signals at 3.34, 4.94 and 5.00 turned into three doublets with *J* = 4.3, 2.3 and 2.3, respectively). ^{13}C NMR: δ 16.13, 19.53 (CH(CH_3)$_2$); 31.01 ($CH(CH_3)_2$); 42.88 (4-C-isox.); 52.87 (OCH_3); 54.66 (2′-C); 60.04 (2-C); 69.46 (3-C); 83.09 (5-C-isox.); 125.87, 128.28, 128.71, 140.13 (Ph); 158.30, 169.89, 175.15 (C=N, C=O ester and amide). MS-FAB$^+$ (*m*/*z*): 364 (MH^+). IR (nujol): 3340 (ν_{OH}, ν_{NH}, OH, NH_2), 1748 ($\nu_{C=O}$, C=O), 1664 ($\nu_{C=N}$, C=N).

4.18. (2*R*)-Amino-[(3*R*)-hydroxy-3-(5*R*)-5-phenyl-4,5-dihydro-isoxazol-3-yl]-propionic acid methyl ester 14

Oil (56%); R_f = 0.39 (ethyl acetate/methanol = 95:5); $[\alpha]_D^{20} = -99.7$ (*c* 0.30, $CHCl_3$). ^{1}H NMR: δ 2.45 (3H, broad m, OH, NH_2); 3.03 (1H, dd, *J* = 17.3, 8.5, 4-H isox.); 3.58 (1H, dd, *J* = 17.3, 11.0, 4-H isox.); 3.78 (3H, s, OCH_3); 4.03 (1H, m, 2-H); 4.71 (1H, d, *J* = 2.9 3-H); 5.60 (1H, dd, *J* = 11.0, 8.5, 5-H isox.); 7.22–7.55 (5H, m, Ph). ^{13}C NMR: δ 43.13 (4-C-isox.); 52.60 (OCH_3); 56.24 (2-C); 68.55 (3-C); 82.41 (5-C-isox.); 125.83, 128.23, 128.72, 140.57 (Ph); 158.87, 172.84 (C=N, C=O). MS-FAB$^+$ (*m*/*z*): 265 (MH^+). IR (nujol): 3435 (ν_{OH}, ν_{NH}, OH, NH_2), 1723 ($\nu_{C=O}$, C=O), 1641 ($\nu_{C=N}$, C=N).

4.19. (2*R*)-[(2*R*)-Amino-3-methyl-butyrylamino]-(3*R*)-hydroxy-3-[(5R)-5-phenyl-4,5-dihydroisoxazol-3-yl]-propionic acid methyl ester 15

Oil (34%); R_f = 0.14 (ethyl acetate/methanol = 95:5); $[\alpha]_D^{20} = -35.3$ (*c* 0.15, $CHCl_3$). ^{1}H NMR: δ 0.80, 0.97 (6H, 2d, *J* = 6.9, CH(CH_3)$_2$); 2.24 (1H, m, $CH(CH_3)_2$); 2.00–2.40 (3H, broad, OH, NH_2); 3.00–3.60 (3H, m, 4-H isox. and 2′-H); 3.80 (3H, s, OCH_3); 4.90 (1H, dd, *J* = 8.7, 3.0, 2-H); 4.99 (1H, broad d, *J* = 3.0, 3-H); 5.61 (1H, t, *J* = 10.4, 5-H isox.); 7.25–7.40 (5H, m, Ph); 8.10 (1H, d, *J* = 8.7, NH–CO); (by deuteration the signals at 2.00–2.40 and 8.10 disappeared and the signals at 4.90 and 4.99 turned into two doublets with *J* = 3.0 and 3.0, respectively). ^{13}C NMR: δ 16.15, 19.66 (CH(CH_3)$_2$); 30.84 ($CH(CH_3)_2$); 42.30 (4-C-isox.); 52.98 (OCH_3); 54.63 (2′-C); 60.13 (2-C); 69.44 (3-C); 83.51 (5-C-isox.); 126.31, 128.43, 128.69, 140.22 (Ph); 158.34, 170.07, 175.32 (C=N, C=O ester and amide). MS-FAB$^+$ (*m*/*z*): 364 (MH^+). IR (nujol): 3387 (ν_{OH}, ν_{NH}, OH, NH_2), 1743 ($\nu_{C=O}$, C=O), 1658 ($\nu_{C=N}$, C=N).

4.20. (2*S*,3*S*,6*R*)-2-[(2*R*)-2-Amino-3-methyl-butyrylamino]-3,6-dihydroxy-4-oxo-6-phenyl-hexanoic acid methyl ester 16

To a solution of **13** (0.4 mmol, 1 equiv) in 5/1 methanol/water (10 mL), were added boric acid (1.2 mmol, 3 equiv) and a spatula tip of Raney-Ni. The mixture was stirred vigorously under hydrogen for 3 h, and then filtered through Celite. After evaporation of the solvent, the residue was treated with brine and extracted with ethyl acetate (5 × 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Compound **16** was pure enough for the spectroscopic and analytical characterisation.

Amorphous solid (55%); $[\alpha]_D^{20} = +60.6$ (*c* 0.42, $CHCl_3$). ^{1}H NMR: δ 0.81, 0.96 (6H, 2d, *J* = 6.9, CH(CH_3)$_2$); 2.25 (1H, m, $CH(CH_3)_2$); 2.20–2.70 (4H, broad, 2OH, NH_2); 3.10–3.21 (3H, m, 5-H and 2′-H); 3.81 (3H, s, OCH_3); 4.79 (1H, broad d, *J* = 1.8, 3-H); 5.16 (2H, m, 2-H and 6-H); 7.20–7.40 (5H, m, Ph); 7.90 (1H, broad d, *J* = 9.1, NH–CO). ^{13}C NMR: δ 16.00, 19.54 (CH(CH_3)$_2$); 30.91 ($CH(CH_3)_2$); 47.24 (5-C); 53.04 (OCH_3); 53.54 (2′-C); 60.00 (2-C); 70.25 (6-C); 77.56 (3-C); 125.58, 127.86, 128.60, 142.64 (Ph); 169.47, 174.79, 208.51 (C=O ester, ketone and amide). MS-FAB$^+$ (*m*/*z*): 367 (MH^+). IR (nujol): 3355 (ν_{OH}, ν_{NH}, OH, NH_2), 1744, 1723, 1663 ($\nu_{C=O}$, C=O ketone, ester, amide).

4.21. Single crystal X-ray structural determination of 8

The intensity data for **8** were collected on a Bruker Smart Apex CCD area detector using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Data reduction was made using SAINT programs; absorption corrections based on multiscan were obtained by SADABS.[28] The structures were solved by SIR-92[29] and refined on F^2 by full-matrix least-squares using SHELXL-97.[30] All the non-hydrogen atoms were refined anisotropically, hydrogen atoms were included as 'riding' and not refined. The ORTEP-III program was used for molecular diagrams.[31]

Crystal data and results of the refinement: colourless prism 0.40 × 0.35 × 0.15 mm, M_r = 359.42, orthorhombic, space group $P2_12_12_1$, *a* = 7.0333(7) Å, *b* = 11.112(1) Å, *c* = 24.773(3) Å, *V* = 1936.1 (3) Å^3, *Z* = 4, *T* = 293(2) K, μ = 0.087 mm^{-1}. 27,891 measured reflections, 2541 independent reflections, 2195 reflections with $I > 2\sigma(I)$, $3.28 < 2\theta < 55.00°$, R_{int} = 0.0241. Refinement on 2541 reflections, 242 parameters. Flack parameter[32] for determination of the absolute configuration = −3.0(13). Final *R* = 0.0366, *wR* = 0.0957 for data with $F^2 > 2\sigma$ (F^2), *S* = 1.069, $(\Delta/\sigma)_{max}$ = 0.001, $\Delta\rho_{max}$ = 0.103, $\Delta\rho_{min}$ = −0.118 eÅ^{-3}.

Crystallographic data (excluding structure factors) for **8** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 736008. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

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