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Asymmetric Deuteration of N-Acetyl-(*Z*)-α,β-Dehydrotryptophan-(*L*)-Phenylalanine Methyl Ester Produced by (*L*)-Tryptophan 2',3'-Oxidase from *Chromobacterium violaceum*. A New Route for Stereospecific Labelling of Peptides.

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Abstract: A novel approach to the synthesis of labelled (^{2}H, ^{3}H) peptides through the catalytic asymmetric reduction of Δ^ZTrp containing peptides, using rhodium complexes with chiral diphosphine ligands as the catalysts, is described. Ac-Δ^ZTrp-(*L*)-Phe-OMe is used as a model substrate to study this new route. The (Z)-α,β-dehydropeptide is produced by (*L*)-tryptophan 2',3'-oxidase from *Chromobacterium violaceum* in a single step reaction. Diastereomeric excesses up to 98 % have been obtained with (*R,R*)-dipamp as ligand in the catalyst. Extremely high stereoselectivities for producing the (*L,L*)- or (*D,L*)-isomer could be achieved using the appropriate chiral ligands. This method has good potential for stereospecific labelling (deuteration or tritiation) of peptides.

INTRODUCTION

Elucidation of peptide structure by NMR, molecular dynamics or peptide-ligand relationship studies, all depend on the achievement of an appropriate isotopic labelling of a peptide.[1] In this domain, improvements could be expected from the regio- and stereoselective insertion of tritium at a specific position in the peptide. Furthermore, if tritium could be introduced directly into peptides, such an approach would give large advantages since this strategy can keep the amounts of radioactive side products at minimum level in sharp contrast with the stepwise synthesis of the labelled polypeptides starting from tritiated amino acids. In this respect, the tritiation of (Z)-α,β-dehydropeptides catalyzed by chiral rhodium complexes provides an useful approach.[2] However, in most cases, the chemical synthesis of (Z)-α,β-dehydropeptides proceeded through a multistep and low-yield procedure.[3] We show that problems can now be overcome by an appropriate enzymatic synthesis.

We previously showed that (*L*)-tryptophan 2',3'-oxidase, an haemoprotein isolated from *Chromobacterium violaceum* (ATCC 12472), is capable to specifically catalyse the α,β-dehydrogenation of various (*L*)-tryptophan (Trp) derivatives.[4] The steric configuration of the α,β-dehydrotryptophanyl (ΔTrp)

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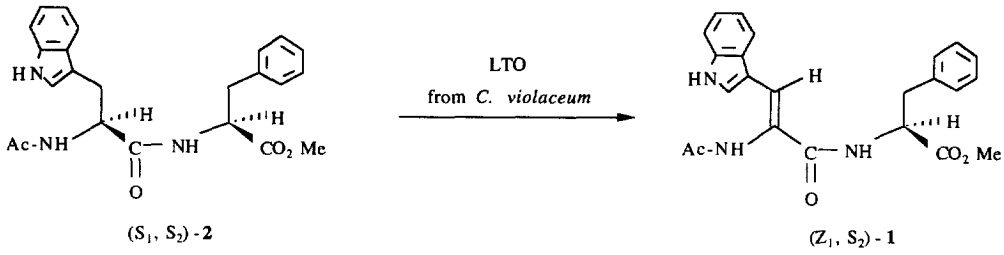
moiety produced by enzymatic route was investigated by ^{1}H-NMR spectroscopy.[5] The (*Z*)-isomer is obtained in pure form. Then, we showed that LTO catalyses the (*Z*)-α,β-dehydrogenation of Trp-side chains in peptides, irrespective of the position of this residue in the sequence.[6] This strategy leads, in a single step reaction, to pure Δ^ZTrp-peptides without side-product formation.[6]

As a result of the aforementioned studies, we proposed a novel approach to the regio- and stereoselective tritiation of proteins by a two-step procedure involving: (i) the enzymatic α,β-dehydrogenation of a (*L*)-tryptophan side chain, and (ii) the asymmetric reduction of the double bond leading to the amino acid residue with either (*D*)- or (*L*)-configuration. This strategy was first applied to the stereoselective deuteration and tritiation of *N*-acetyl-Δ^Ztryptophanamide.[7] We showed that the asymmetric tritiation of this compound catalysed by the cationic complex [Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^- in methanol at room temperature and under atmospheric pressure gave the corresponding *L*-isomer with at least 92% enantiomeric excess (e.e.).[7] However, these preliminary data suggested that this approach was found to be limited to labelling of amino acids and asymmetric deuteration or tritiation of Δ^ZTrp peptides could not be obtained in good yields under standard conditions and would require an higher deuterium or tritium pressure. Recently, we developped a new automatic gas transfer unit supplied with a liquid helium cryostat that allowed us to reach a deuterium or tritium pressure higher than 20 atm. Therefore, our new route for stereospecific labelling of peptides should be extended without problems to the synthesis of stereospecifically labelled peptides and proteins.

In connection with the regiospecific and stereoselective labelling of polypeptides, we will give here a full account of our research on the asymmetric hydrogenation and deuteration under elevated pressure of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1** produced by L-tryptophan 2',3'-oxidase. We used hydrogenation and deuteration in the subsequent experiments as a model for the tritiation reaction. Efficiency of chiral diphosphine ligands as well as the effect of the pressure on the catalytic asymmetric induction will be discussed in terms of stereoselectivity.

RESULTS AND DISCUSSION

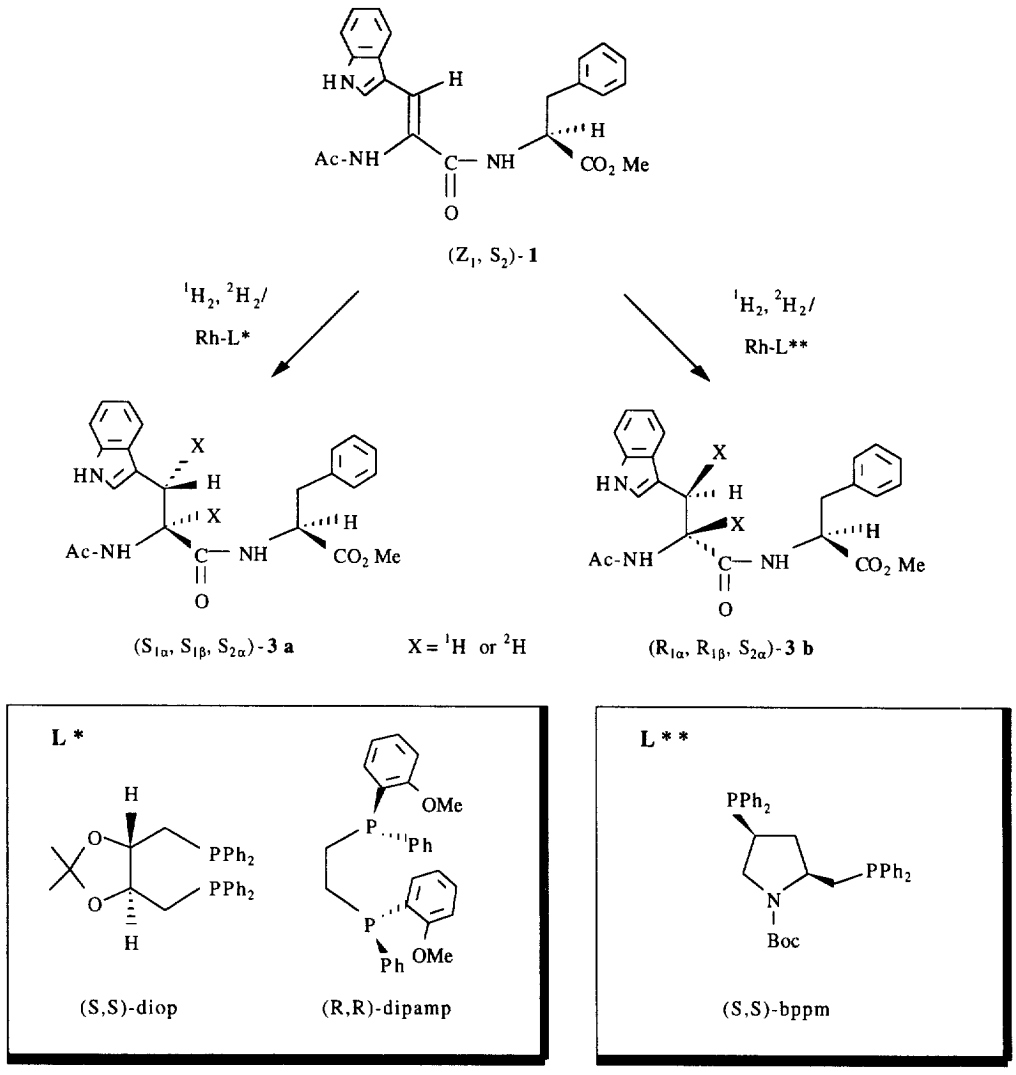
Ac-Δ^ZTrp-(*L*)-Phe-OMe **1** was synthesized by enzymatic α,β-dehydrogenation of Ac-(*L*)-Trp-(*L*)-Phe-OMe **2** using the (*L*)-tryptophan 2',3'-oxidase from *Chromobacterium violaceum*. The compound **1** was obtained in quantitative yield and purified by HPLC (70% yield, Scheme 1).



Scheme 1

Asymmetric micro-hydrogenation and micro-deuteration under pressure of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1** (2.5 mg, 6 μmol) were run using an automatic gas transfer unit[8] supplied with a liquid helium cryostat and fitted with an inlet for introduction of the solvent. The reduction flask was adapted to special experimental conditions (low sample weigth and high pressure). Asymmetric reductions of **1** were performed in methanol in the presence of rhodium complexes containing various chiral diphosphines such as (*S,S*)-diop,[9] (*S,S*)-bppm[10] or (*R,R*)-dipamp[11] (Scheme 2). With the exception of Rh/(*R,R*)-dipamp catalyst, which was used as the isolated cationic complex ([Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^-), all other catalytic systems were generated in situ by mixing the ligand with the dimeric complex [Rh (COD) Cl]$_2$ just before the use. The three chiral diphosphines, (*S,S*)-diop, (*S,S*)-bppm and (*R,R*)-dipamp, are known to give efficient stereoselective rhodium catalysts[12] and are used here as standard chiral ligands. For the purpose of comparison, we examined the enantioselectivity associated with these ligands in rhodium catalysed reductions involving dehydropeptide **1**. The main results are listed in Table 1.

Under 4 atm of hydrogen pressure at room temperature the hydrogenation of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1** proceeded smoothly, leading to the corresponding dipeptide analog **3** in quantitative yield. The diastereomeric excesses were in the range of 54 to 82%. The (*S,S*)-diop ligand and the (*S,S*)-bppm diphosphine did not give high diastereoselectivity. With the in situ neutral complex [Rh Cl (*S,S*)-diop], the asymmetric hydrogenation of **1** in methanol at room temperature and 4 atm of hydrogen gave Ac-(*L*)-Trp-(*L*)-Phe-OMe **3a** with 54% d.e. (entry 1). Ac-(*D*)-Trp-(*L*)-Phe-OMe **3b** with 72% d.e. was obtained using [Rh Cl (*S,S*)-bppm] catalyst in methanol at room temperature and 4 atm of hydrogen (entry 2). As already demonstrated for the asymmetric hydrogenation of dehydropeptides,[13] the best results were obtained when the cationic complex [Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^- was used as the chiral catalyst. The reduction proceeded smoothly at room temperature and 2 atm of hydrogen to give Ac-(*L*)-Trp-(*L*)-Phe-OMe **3a** with high stereoselectivity (82% d.e., entry 3).



Scheme 2

As for the reaction rate, the asymmetric reduction of **1** was found to be slower than that of *N*-acetyl-Δ^Ztryptophanamide.[7] Thus, gentle pressurization (4 atm) was very efficient to promote the reaction smoothly. Depending on catalyst structure, the relative rate of the reaction decreases in the order (*S,S*)-bppm - (*S,S*)-diop >> (*R,R*)-dipamp. In the case of complex [Rh (*R,R*)-dipamp (COD)]$^+BF_4^-$, the reduction reaction at higher hydrogen pressure (18 atm) has also been examined. After only 4 h of reaction,

the reduction was quantitative (entry 4). Regarding the enantioselectivity dependence on hydrogen pressure, it is interesting to observe that an increase in pressure resulted in improved enantioselectivity (82% and 98% d.e. respectively at 4 to 18 atm, entries 3 and 4). This is in contrast with the generally well-established inverse dependence of enantioselectivity on hydrogen pressure.[14] However, a few examples are also known where increasing hydrogen pressure leads to an improvement of selectivity.[15]

Table 1. Asymmetric hydrogenation and deuteration of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1**.[a]

Entry	Catalyst (ligand)[b]	Pressure (atm)	Time (h)	Diastereomer ratio[c] *LL/DL*	d.e. (%)
1	(*S,S*)-diop	4	4	77/23	54
2	(*S,S*)-bppm	4	4	14/86	72
3	(*R,R*)-dipamp	4	18	91/9	82
4	(*R,R*)-dipamp	18	4	99/1	98
5	(*S,S*)-diop	4[d]	4	79/21	58
6	(*S,S*)-bppm	4[d]	4	13/87	74
7	(*R,R*)-dipamp	18[d]	4	>99/<1	>98

a) Reductions were performed in near-quantitative conversion (no starting material detected by HPLC). [substrate]/[Rh] = 1.5; [substrate] = 0.006 M in methanol. Reaction performed at room temperature.
b) The ligands are : (*S,S*)-diop : 4,5-bis-diphenylphosphino-2,2-dimethyl-1,3-dioxolane; (*S,S*)-bppm : *t*-butyl-4-diphenylphosphino-2-[(diphenylphosphino)methyl]pyrrolydine-1-carboxylate; and (*R,R*)-dipamp : 1,2-bis[(2-methoxyphenyl)phosphino]ethane.
c) Measured by HPLC. The configuration of the new asymmetric center was assigned by HPLC as compared to reference compounds.
d) Reaction performed with D_2 instead of H_2.

With regard to the regiospecific and stereoselective labelling of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1**, we carried out the dideuteration of **1** using [Rh Cl (*S,S*)-diop], [Rh Cl (*S,S*)-bppm] and [Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^- as catalyst. The in situ neutral complex [Rh Cl (*S,S*)-diop] leads to a complete reduction giving a moderate excess of Ac-(*L*)(2,3-^{2}H)-Trp-(*L*)-Phe-OMe **3a** (58% d.e., entry 5). However, higher chiral recognition was found using the cationic complex [Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^- (>98% d.e., entry 7). In the presence of (*S,S*)-bppm as a ligand of rhodium, the asymmetric deuteration of **1** gave Ac-(*D*)(2,3-^{2}H)-Trp-(*L*)-Phe-OMe **3b** with 74% d.e. (entry 6). The diastereomeric ratios were higher than observed with hydrogen. Several factors (e.g. kinetic and thermodynamic parameters of the reactions, role of solvent, nature of the rhodium catalyst) can be responsible for the different behaviour with hydrogen and deuterium.

CONCLUSIONS

The stereoselective reduction of Δ^ZTrp containing peptides produced by (*L*)-tryptophan 2',3'-oxidase coupled with chiral rhodium-diphosphine complexes is a powerful strategy for the regioselective and stereoselective labelling (^{2}H) of peptides. We demonstrated that optically pure (*L,L*)- or (*D,L*)-dipeptides (**3a** or **3b**) can be readily synthesised with high diastereomeric excesses by suitable choice of the chiral catalyst. The cationic complex [Rh (*R,R*)-dipamp (COD)]$^+$ BF_4^- possesses high efficiency for the asymmetric reduction of Ac-Δ^ZTrp-(*L*)-Phe-OMe **1**, giving Ac-(*L*)-Trp-(*L*)-Phe-OMe **3a** with diastereoselectivities up to 98 %. In addition, it was shown that hydrogen pressure had a remarkable effect on the stereoselectivity of the reaction. Increasing hydrogen pressure contributes, in the present case, to the enhancement of the diastereoselectivity.

Starting from a (*L*)-tryptophanyl containing peptide, the proposed novel strategy is a powerful and readily applicable method for the asymmetric synthesis of peptide which involve tryptophanyl moiety with unnatural configuration.

These experiments should be extended without problems to the synthesis of stereospecifically tritiated peptide **1**. Further studies on the application of this methodology to the asymmetric syntheses of labelled (^{2}H, ^{3}H) peptide hormones are under progress.

EXPERIMENTAL SECTION

General Methods

^{1}H-NMR spectra were recorded in methanol-d_4 using a Brüker WM 250 spectrometer (250 MHz). Chemical shifts are reported in δ values with TMS as an internal reference. Optical rotation was measured on a Perkin Elmer Polarimeter 241. HPLC analyses were performed on a Waters 600 chromatographic system (Hypersil ODS column 250 x 4.6 mm) supplied with a Waters 996 photodiode array detector. Ultraviolet spectra were recorded with a Beckman DU-70 spectrophotometer. Products were analyzed either by chemical ionization-mass spectrometry (Nermag R 10-10).

Methanol was freshly distilled, dried following literature method and stored under argon. All experiments with organometallic elements were performed using standard Schlenk techniques.

Enzymatically prepared Ac-Δ^ZTrp-(*L*)-Phe-OMe 1

(*L*)-Tryptophan 2',3'-oxidase was purified from *C. Violaceum* (ATCC 12472) according to Genet and coworkers.[2] 1 mM Ac-(*L*)-Trp-(*L*)-Phe-OMe **2** (10 mg, 0.025 mmol) was incubated in 50 mM succinate buffer, pH 5.6, containing 40 μg ml^{-1} catalase, in the presence of 1.4 nM enzyme, for 15 h at 30 °C. The α,β-

dehydrogenation reaction was followed spectrophotometrically by monitoring the UV-spectrum of **1**. The α,β-dehydro product **1** showed a characteristic absorption peak at λ_{max} = 336 nm. It was purified on reverse-phase chromatography (Hypersil ODS column; eluent, 50% methanol in water; flow rate, 1 ml min^{-1}; retention time, 23.5 min; UV detection at 280 nm). Evaporation of the solvent afforded 7 mg (70%) of **1** as a yellow oil. The dehydropeptide **1** was then characterized by mass spectrometry (m/z = 406, MH^+).
^{1}H-NMR (Me_2SO-d_6) δ : 11.60, (broad s, 1H, indole NH), 9.10, (broad s, 1H, amide NH), 7.75, (m, 2H), 7.45, (m, 2H), 7.30, (m, 5H), 7.25, (m, 2H), 4.40, (t, J = 8 Hz, 1H), 3.60, (s, 3H), 3.30, (m, 2H), 2.00, (s, 3H).

Preparation of chiral catalyst solution

[Rh Cl (*S,S*)-bppm] and [Rh Cl (*S,S*)-diop] were prepared *in situ* according to published methods[6], by reaction of [Rh Cl (COD)]$_2$ with chiral diphosphine in degassed methanol. Typically, [Rh Cl (COD)]$_2$ (2 μmol) and (*S,S*)-bppm (4 μmol) were dissolved in 1 ml of methanol under argon. The solution was then stirred for 30 min. The complex thus obtained was ready to use for asymmetric reduction and was introduced into the hydrogenation flask by means of a syring. Any contact with air was avoid.

Reduction, general procedure

The solution of appropriate catalyst (4 μmol) in degassed methanol (1 ml) was added under argon to the dehydropeptide **1** (6 μmol) in the hydrogenation flask which was connected to the reduction apparatus. The argon atmosphere was replaced with hydrogen or deuterium. The reductions were run under the reaction conditions given in the preceding table. The solvent was removed under reduced pressure. Conversion rates were determined by HPLC analysis.

Identification of diastereomers

The stereoselectivity was measured by HPLC on a Hypersil ODS column (250 x 4.6 mm) using methanol-water (50/50) eluent. The flow rate was 1 ml/min. with UV detection at 280 nm. The two resultant diastereomers **3a** and **3b** were characterized as compared to reference compounds. Retention times were : 24.5 min for the (*L,L*)-diatereomer **3a** and 29.5 min for the (*D,L*)-diastereomer **3b**.

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