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ASYMMETRIC TRITIATION OF N-ACETYL α-β DEHYDROTRYPTOPHANAMIDE

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SUMMARY

Rhodium-DiPAMP complex catalyzes the stereoselective addition of two tritium atoms on N-acetyl -α-β dehydrotryptophanamide. The substrate was prepared by dehydrogenation of N-acetyltryptophanamide with TSO (Tryptophan-Side Chain oxidase) from Pseudomonas. Tritiated N-acetyl-tryptophanamide was obtained with the theoretical specific radioactivity (58 Ci/mmole). The enantiomeric excess of the L-diasteroisomer reached 94.4 %. ^3H NMR spectra indicated the selectivity of ^3H-labelling on the C_α - C_β double bond. This new approach of tritiation by homogeneous catalysis applied to tryptophanyl-containing peptides is discussed.

Key words : asymmetric - tritiation - tryptophan - dehydrogenase - TSO.

1 - INTRODUCTION

Highly active homogeneous catalysts were discovered by Wilkinson in 1966 (1). Spectacular progress has been made with chiral rhodium diphosphines which induce a high enantiomeric excess (2,3). Unsaturated α-β aminoacids containing peptides used as precursors can be very helpful for tritium labelling. The eminent efficiency of stereospecific catalysts in the case of dehydrophenylalanyl residue included in a dipeptide (4,5) lead us to label the tryptophanyl residue. As a model, N-acetyl -α-β dehydrotryptophanamide (Δ NATA) prepared by the dehydrogenation of (NATA) with TSO (Tryprophan-Side Chain oxidase) extracted from Pseudomonas, was asymetrically tritiated in presence of the Rhodium (DiPAMP) complex, a chiral catalyst. The ultimate aim of the work was to extend this method to α- β dehydrotryptophanyl - containing peptides.

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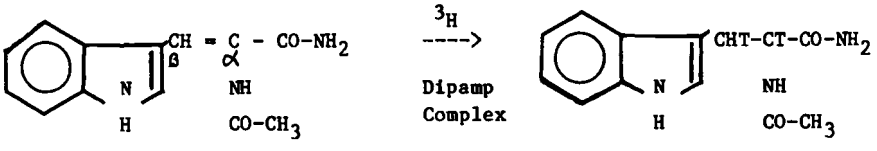
2 - MATERIALS

The cationic catalyst [Rh (1,5 - cyclooctadiene) DiPAMP]$^+$ BF_4^- was a generous gift of Dr. Koenig (Monsanto Co, USA). Pure tritium gas was made by Commissariat à l'Energie Atomique (Saclay, France). All reagents were of the highest purity. Precoated silicagel plates SiF_{254} for thin-layer chromatography (TLC) were obtained from Merck (W. Germany), G 1500 from Schleicher Schull (W. Germany), P_2 resin (100-200 mesh) from Biorad (USA), Servachrom XAD.2 resin from Serva (W. Germany). The following solvent systems were used for TLC : (a) chloroform/ethanol (65 : 35, v/v) and (b) chloroform/isopropyl ether/acetic acid/ethanol (85 : 7 : 5 : 20, v/v) and (c) ethyl/acetate/pyridine/water/ acetic acid (30 : 10 : 7 : 3, v/v - upper phase). N-Acetyl-L-Tryptophanamide (L-NATA), N-Acetyl-L-Tryptophan (L-NAT) and δ- Chymotrypsin were supplied by Sigma (USA).

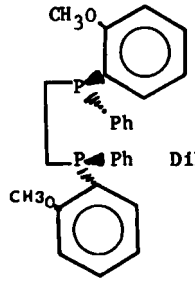
The automatic gas transfer unit used for catalytic tritiation was built by Morgat, Desmares and Cornu and was described elsewhere (11). Absorption spectra were obtained with a Cary 210 (Varian). NMR spectra of tritiated NATA were recorded on a Brücker WP-100 spectrometer operating at 106 MHz. Tritium determinations were made in a SL 30 Intertechnique liquid scintillation counter.

SCHEME

Asymmetric tritiation of N-acetyl - α-β dehydrotryptophanamide



N-acetyl -α-β dehydrotryptophanamide N-acetyl-tryptophanamide



DiPAMP =

The chiral catalyst is a Dipamp complex :
[Rh-(COD) Dipamp]$^+BF_4^-$ COD
COD = cycloocta- 1,5 - diene

(-) [1,2 - bis (O-anisylphenylphosphino) ethane]

Radioscans of TLC were performed with a Berthold Scanner II. Autoradiography of TLC was achieved on X-O mat R films (kodak).

Liquid chromatography was effected using a LKB 11300 equipped with a peristaltic pump (LKB). The elution profile was recorded with a Gilson analyser system.

3 - METHODS

3.1 - Preparation of N-acetyl α-β-dehydrotryptophanamide

- Strain and media

Pseudomonas cells (ATCC 29574) were grown as described (6) in Nutrient Broth medium at 30° C, centrifuged 48 hours after the beginning of stationary phase. Then, the pellet of bacteria was kept at - 70° C. These cultures were prepared in the laboratory of Dr. Scandelari (Chimie bactérienne, CNRS, Marseille).

- Preparation of the enzyme extract

The manipulations were carried out at 0° - 4° C. Frozen bacteria (1.3 g) were suspended in 50 mM potassium phosphate buffer pH 6.8 (2.6 ml). The cells were disrupted by sonication during 1.5 min. (30 sec. periods) (20-200 S - Bioblock Scientific Sonifer). The crude extract was acidified at pH 3 with formic acid. This step eliminates cellular debris and mostly acidic proteins. The enzymatic activity remained in solution. The 30,000 x g supernatant was adjusted to pH 6.8 with 4M NH_4OH and directly used or precipitated by 60 % ammonium sulfate. The pellet was suspended in phosphate buffer and kept at 0° C.

- Enzyme assay

The α-β desaturating activity of enzymatic fractions were measured on L-NATA by spectroscopy (8). The reaction which is pH dependent was performed at 21° C, pH 6.8 (pH value leading predominantly to ΔNATA, (8)). The incubation mixture contained 50 mM phosphate buffer pH 6.8 (1 ml) and 0.5 mM NATA (20 µl) ; the reaction was initiated by the addition of enzyme (10 µl). The ΔNATA formation was monitored by the increase in absorbance at 333 nm ($\varepsilon^{H_2O}_{mM}$: 19.8 cm^{-1}). The enzymatic specific activity (nmoles substrate/min/mg protein) was shown to be 30 times higher in the pH 6.8 supernatant than in the crude extract. Protein determinations were made by colorimetric reaction (12).

- Preparation of NATA

A solution of NATA (100 µl, 0.5 mM) in 100 mM phosphate buffer pH 6.8

(1 ml) was incubated at 21° C with enzyme extract (50 ul) until complete desaturation. NATA was freed of proteins by chromatography (P2 column : 0.8 x 26 cm). Elution was made with 5 % aqueous acetic acid (flow rate : 0.1 ml/min). The peak showing a maximum absorption at 333 nm was lyophilized and solubilized in 1 ml methanol. The purity of the product was tested by spectrophotometry and TLC (Methods).

3.2 - Tritiation of NATA by homogeneous catalysis

- Tritiation

A special vial was previously adapted to the tritiation conditions of homogeneous catalysis (5). Nitrogen and solvents were injected through a lateral septum. Between each introduction of nitrogen or tritium, a vacuum of 10^{-4} Torr was reached. The vial containing ΔNATA (2 μmoles) dissolved in methanol (0.5 ml) was connected under nitrogen to the tritiation apparatus. Dipamp complex catalyst (0.8 μmole) dissolved in methanol (0.5 ml) was added. The solution was frozen. Tritium gas was introduced and compressed until 1.15 bars. After thawing, the reaction mixture was kept at 40° C and magnetically stirred 3 hrs. The labile tritium atoms were removed by successive flash evaporations with methanol (150 ml).

- Purification of tritiated NATA

In a first step a large amount of catalyst (80 %) was separated from the tritiated product by differential solubility. The tritiation mixture was lyophilized and solubilized in 5 % aqueous acetic acid. In a second step, the remaining catalyst was displaced from the catalyst complex-substrate with dimethyl acetamide (DMA) : (1 %, v/v). The catalyst was retained on XAD - 2 resin (1.2 x 10 cm) by differential adsorption. The elution was made using a linear gradient of methanol/water 70 % - 100 % containing 1 % DMA (elution rate : 0.2 ml/min). A small percentage (5 %) of catalyst was associated with the tritiated product. The radioactive fractions corresponding to ^{3}H-NATA were collected.

- Determination of the specific radioactivity of ^{3}H-L-NATA :

The specific radioactivity of ^{3}H-L-NATA was obtained from the absorption value at 280 nm (ε_M : 5850) and radioactivity counting of an aliquote fraction.

3.3 - Determination of the enantiomeric excess of L-NATA

The determination of enantiomeric excess of L-NATA was made by enzy-

matic deamidation with δ-Chymotrypsin. The reaction mixture consisted of : L-NATA (1 μmole), ^{3}H NATA (8 μCi), 50 mM Tris/HCl buffer containing 100 mM $CaCl_2$ (pH 8, 100 μl), and 1 mM δ-Chymotrypsin (20 μl) dissolved in 1 mM HCl. After complete reaction (48 hrs, 37° C), the incubation mixture was then analyzed by TLC in system c (Methods) [Rf. : L-NATA : 0.66, L-NAT : 0.49]. The optical yields of L-NATA and D-NATA were deduced from the radiochromatogram. The enantiomeric excess of L-NATA was calculated by the formula : (L-D)/(L + D) x 100.

4 - RESULTS

The introduction of two tritium atoms (58 Ci/mmole) on the C_α - C_β bond in ΔNATA as model was successfully realized by reduction using homogeneous catalyst. Tritiation with Rhodium-DIPAMP complex led to a high enantiomeric excess of L-NATA : 94.4 %. In addition, the reduction yield reached 100 % using our conditions. ^{3}H-NMR spectroscopy showed the specific location of tritium atoms in C_α (48 %) and C_β (52 %). This labelling also confirms the insertion of a double bond at C_α - C_β using TSO . An easy partial purification of TSO was perfected in this work. The complete solubility of the enzyme in 1 M formic acid allowed us to develop a rapid preparation method. The supernatant pH 6.8, (purification factor : 30, yield : 40 %) enriched in TSO activity revealed the presence of the two isoenzymes TSO I and TSO II (unpublished results) described by Takaï (9, 10).

5 - DISCUSSION

The tritium labelling performed on a model compound : N-acetyl-α - β - dehydrotryphanamide was stereoselective and the specific radioactivity achieved was theoretical. These promising data prompted us to tritate tryptophan-containing peptides. According to some results (13, 14), preliminary assays with hydrogen showed than the application to larger peptides requires higher pressures 10-20 atm). A new tritation apparatus adapted to these conditions is now under study.

The separation of catalyst from tritiated peptide was partially achieved, (5 % only remained). It is well known that the dehydrosubstrate is linked to catalyst during the reaction (15). After reduction, the saturated compound could be probably complexed by its carboxyle group or its amide function. Dimethylacetamide displaced efficiently the catalyst but the use of other ligands is presently examined.

In order to tritiate peptides on different aminoacid residues, the tritiation of dehydrosubstrates can be associated to other tritiation methods (16). With the same objective, we are searching for enzymes similar to TSO, for intro-

ducing a $C_\alpha - C_\beta$ bond in different aminoacids. Particularly, the work of Davis et al. (17) prompted us to isolate from Chromobacterium violaceum another enzyme catalyzing the dehydrogenation of the alanine side chain tryptophan. This enzyme possesses an activity and a specificity different from those of TSO. The two enzymes seem to be complementary about their action on tryptophanyl-containing peptides (unpublished results). Not any decarboxylation by C. violaceum enzyme has been observed upon free carboxyle-peptides.

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