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STEREOCHEMICAL ASPECTS OF THE CONVERSION OF CYCLOPEPTINE INTO DEHYDROCYCLOPEPTINE BY CYCLOPEPTINE DEHYDROGENASE FROM *PENICILLIUM CYCLOPIUM*

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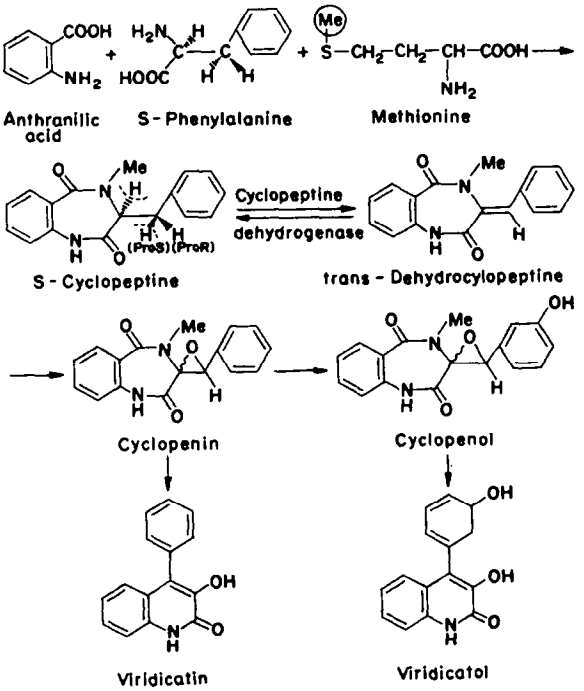
Key Word Index—*Penicillium cyclopium*; Aspergillaceae; Ascomycetes; biosynthesis; stereochemistry; cyclopeptine dehydrogenase; benzodiazepine alkaloids; cyclopeptine; dehydrocyclopeptine.

Abstract—Cyclopeptine dehydrogenase, an enzyme from *Penicillium cyclopium*, catalyses the reversible transformation of the benzodiazepine alkaloids cyclopeptine and dehydrocyclopeptine. By the dehydrogenation of cyclopeptine two hydrogen atoms are removed from the positions 3 and 10. It was demonstrated that, from the two optical isomers of cyclopeptine, only the naturally occurring 3*S*-compound was used as substrate by cyclopeptine dehydrogenase. To test the stereospecificity of the enzyme with respect to the second hydrogen which is eliminated from C-10 a mixture of cyclopeptine-3*S*-[10*R*-$^3H_1$] and cyclopeptine-3*R*-[10*S*-$^3H_1$] was prepared. The 3*S*-isomer was transformed by the enzyme into radioactively labelled dehydrocyclopeptine. This demonstrated that cyclopeptine dehydrogenase removes the 10-pro*S* hydrogen atom from the cyclopeptine molecule. Because the formed dehydrocyclopeptine has the *trans*-configuration it is probable that a synperiplanar elimination takes place. The hydride ion removed from cyclopeptine is transferred to the 4-pro*R*-position of NAD^+. Cyclopeptine dehydrogenase thus belongs to the A-specific dehydrogenases.

INTRODUCTION

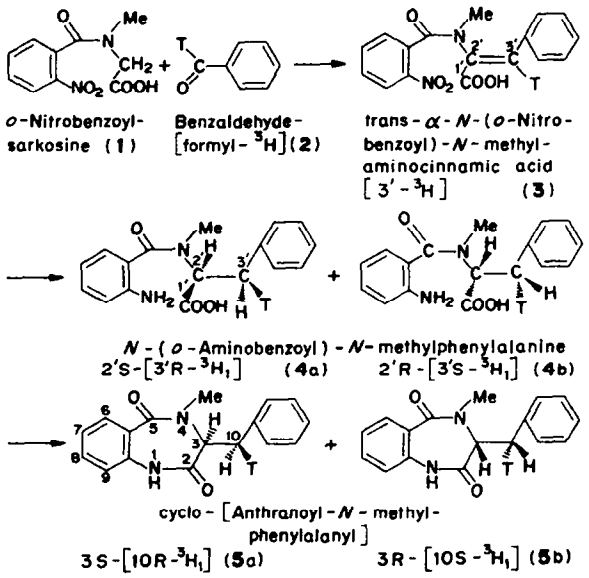
Emerged cultures of *Penicillium cyclopium* produce during the idiophase benzodiazepine alkaloids of the cyclopenin group [1]. The biosynthesis of the major alkaloids of this type, cyclopenin and cyclopenol, proceeds via the cyclic anthranoyl–phenylalanyl derivatives cyclopeptine and dehydrocyclopeptine [2] (Scheme 1). The interconversion of these two compounds is catalyzed by a $NAD(P)^+$-dependent dehydrogenase (cyclopeptine dehydrogenase). This enzyme recently was partly purified and some of its properties were described [3]. During the dehydrogenation of cyclopeptine at positions 3 and 10 two hydrogen atoms are removed, one of which is transferred to $NAD(P)^+$. This paper is concerned with the stereochemical aspects of this reaction with respect to the hydrogen donor as well as to the acceptor molecule.



Scheme 1. Biosynthesis of the benzodiazepine alkaloids in *Penicillium cyclopium*.

RESULTS AND DISCUSSION

Previous experiments have shown that cyclopeptine dehydrogenase is specific only for the naturally occurring 3*S*-isomer of cyclopeptine [3]. From this compound the hydrogen present at position 3 is removed. It was unknown, however, which of the two hydrogen atoms of the CH_2-group at C-10 is removed during the dehydrogenation. To decide between the two possibilities cyclopeptine was prepared which was specifically labelled with tritium at C-10. In this cyclic peptide no isomerization of the phenylalanine moiety takes place (phenylalanine itself is easily racemized by cultures of *P. cyclopium* [4]). Furthermore this compound can be tested by *in vitro* experiments with purified cyclopeptine dehydrogenase preparations which make subsequent modifications of the formed product most unlikely.



Scheme 2. Synthesis of specifically labelled cyclopeptine (cyclo-[anthranoyl-*N*-methyl-phenylalanyl]).

Specifically labelled cyclopeptine was synthesized from benzaldehyde-[formyl-^{3}H](2) by the route outlined in Scheme 2. By condensation with *o*-nitrobenzoyl-sarcosine (1), α-*N*-(*o*-nitrobenzoyl)-*N*-methyl-aminocinnamic acid-[3'-^{3}H] (3) was synthesized. Hydrogenation of this compound with platinium/H_2 gave, by the usual *cis*-addition of hydrogen [5,6], two isomers of labelled *N*-(*o*-aminobenzoyl-*N*-methylphenylalanine (**4a** and **4b**) which by treatment with acid cyclized to a mixture of two isomers of cyclopeptine (cyclo-[anthranoyl-*N*-methyl-phenylalanine]) labelled at position 10 (**5a** and **5b**).

To define the stereochemistry of the cyclopeptine isomers formed the geometry of the cinnamic acid intermediate **3** must be known. Comparison of its NMR-spectrum with data from the literature [7] showed that it was not identical with the *cis*-isomer and that its methyl ester had the *trans*-configuration (Table 1). In the preparation no signals arising from the *cis*-isomer could be observed. This result is in agreement with the formation of *trans*-cinnamic acid and its derivatives by a similar condensation [6,8,9].

Stereochemical purity of the mixtures **4a** and **4b** and **5a** and **5b** requires that, with respect to C-atom 2' of **4** (C-3 of **5**), their synthesis proceeds without racemization. To check this point *N*-(*o*-nitrobenzoyl)-*N*-methyl-*S*-phenylalanine [10] was transformed into cyclopeptine by the experimental conditions used during the preparation of **4a**, **4b**, **5a** and **5b**. Measurement of the optical rotation

Table 2. Transformation of compounds **5a** and **5b** into dehydrocyclopeptine-[10-^{3}H$_1$] by cyclopeptine dehydrogenase

Compound	Cyclopeptine Initial	Cyclopeptine Reisolated	Dehydrocyclopeptine Isolated
Amount (μmol)	10	7.4	2.1
Specific radioactivity (μCi/μmol)	1.3	1.3	1.1
Incorporation			85%

To the solution of 10 μmol of the compounds **5a** and **5b** in 0.2 ml MeOH 20 ml Tris-HCl-buffer (50 mM, pH 9.1) containing 50 mg NAD^+ and, at 1 hr intervals, 8 nkat cyclopeptine dehydrogenase in 1 ml of the same buffer, were added. After 6 hr incubation at 30° the mixture was extracted with ethylacetate. The organic phase was concentrated and the benzodiazepine derivatives were separated by TLC (Kieselgel PF_{254}; toluene–HOAc–EtOH–H_2O, 85:10:5:0.15; development 4 times; R_f-values: cyclopeptine 0.60, dehydrocyclopeptine 0.68). Scanning of the chromatogram demonstrated that cyclopeptine and dehydrocyclopeptine were radioactive. After rechromatography they were quantitated by measuring the UV absorption at 293 nm and 286 nm, respectively.

demonstrated that the product was indeed the stereochemically pure *S*-isomer indicating that the radioactive compounds are also formed without isomerization.

From the two isomers present in the radioactive cyclopeptine preparation only the 3*S*-[10*R*-^{3}H$_1$]-compound (**5a**) is a substrate of cyclopeptine dehydrogenase. The mixture of **5a** and **5b** therefore could be used to test whether the 10*R*-tritium atom is lost by the dehydrogenation reaction. The results in Table 2 showed that, under conditions which gave a high yield of the dehydrogenated product, about 40% of the 3*S*-isomer of cyclopeptine was transformed into dehydrocyclopeptine. The specific activity of the latter compound was 85% of the initial cyclopeptine. The tritium atom of **5a** therefore remained during the dehydrogenation, and thus cyclopeptine dehydrogenase removes the pro*S* hydrogen from C-10.

During the dehydrogenation of cyclopeptine *trans*-dehydrocyclopeptine is formed. It is therefore anticipated that cyclopeptine is held at the surface of the enzyme molecule in the "*trans*-conformation" given in Scheme 1. If this is true the hydrogen atoms at C-3 and C-10 are removed by a synperiplanar elimination (*cis*-elimination). It is noteable that during the formation of mycelianamide from tyrosine-2*S*-[3*R*-^{3}H$_1$] by *Penicillium griseovulvum* a synperiphanar elimination of the 2*S* and 3*S* hydrogen atoms is to be expected, too [11].

By feeding the compounds **5a/b** to emerged cultures of *P. cyclopium* during the idiophase radioactivity was

Table 1. NMR data for α-*N*-(*o*-nitrobenzoyl)-*N*-methyl-aminocinnamic acid methyl esters

Groups	δ-Values: Prepared compound	*trans*-compound after [7]	*cis*-compound after [7]
3H, 3H, *s*, *s'*, N-Me	2.88 and 3.76	2.88 and 3.78	3.20 and 3.38
3H, 3H, *s*, *s'*, O-Me	3.40 and 3.95	3.40 and 3.95	3.59 and 3.72
5H, *m*, C_6H_5	7.25–7.50	7.25–7.60	6.85–7.45
5H, *m*, aromatic H-atoms and vinyl-H	7.68–8.05	7.65–7.90	7.55–8.20

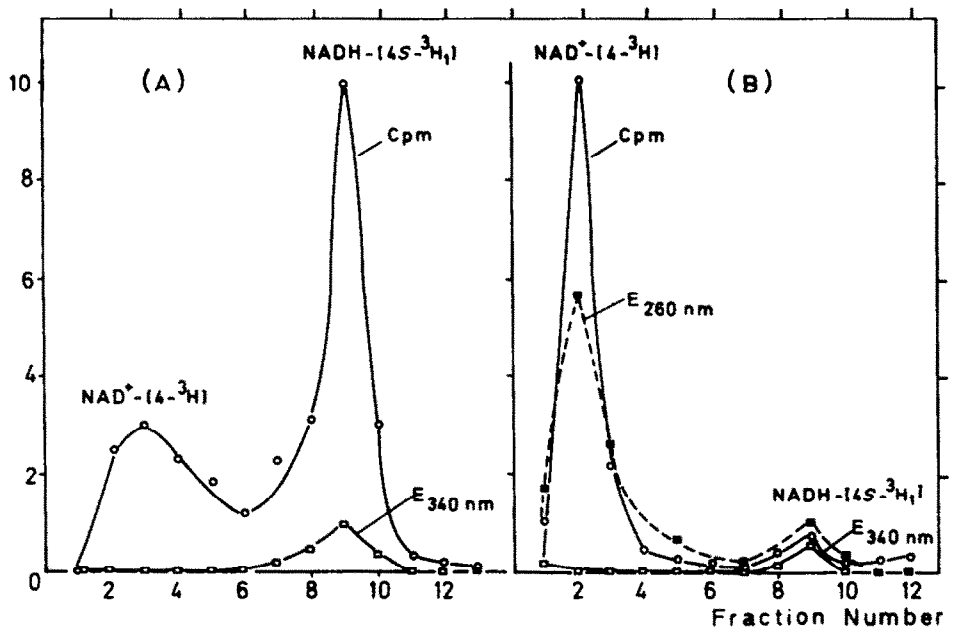
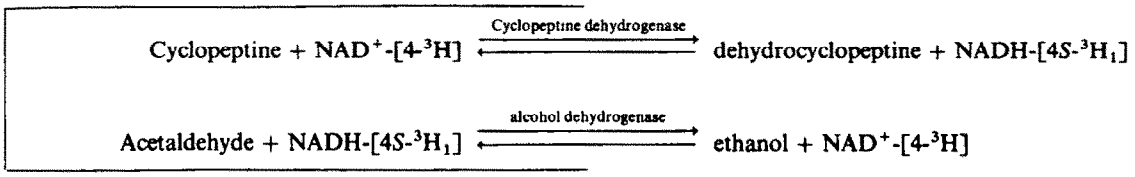


Fig. 1. Stereospecificity of cyclopeptine dehydrogenase with respect to NAD$^+$ (a) Formation of NADH-[4S-$^3H_1$] from NAD$^+$-[4-^{3}H] and cyclopeptine by cyclopeptine dehydrogenase: 0.075 μmol NAD$^+$-[4-^{3}H] (3.75 μCi), 10 μmol *S,R*-cyclopeptine, 4 nkat cyclopeptine dehydrogenase and 125 μmol Tris–HCl-buffer pH 9.1 in 2.5 ml water were incubated 60 min at 30°. After cooling to 0° the mixture was fractionated on a DEAE-cellulose column (1 × 4 cm) as described by Davies *et al.* [16]. (b) Formation of NAD$^+$-[4-^{3}H] from NADH-[4S-$^3H_1$] by alcohol dehydrogenase and acetaldehyde: 0.2 ml of the fraction 9 of experiment (A) (10^5 cpm), 2 μmol unlabelled NADH, 0.1 ml acetaldehyde, 8 μkat alcohol dehydrogenase and 100 μmol Tris–HCl-buffer pH 9.1 in 2 ml H_2O were incubated 30 min at 30°. After cooling to 0° the mixture was fractionated as for Experiment A. Ordinate values: for ○——○ 10 represents 2 × 10^6 cpm in (A) and 6 × 10^4 cpm in (B). respectively; for ■------■ 10 represents an E_{260} nm value of 0.4; for □——□ 10 represents an E_{340} nm value of 0.4.

incorporated not only into dehydrocyclopeptine but also into the benzodiazepine alkaloids derived from it (Scheme 1). This result indicates that the dehydrogenation of cyclopeptine proceeds both *in vivo* and *in vitro* by the same mechanism. It also shows that during the epoxidation of dehydrocyclopeptine (Scheme 1) the hydrogen atom at C-10 is not eliminated. However, if the alkaloids cyclopenin and cyclopenol were transformed into viridicatin and viridicatol respectively, the radioactivity is lost. This agrees with its location at C-10, the hydrogen of which is eliminated during the formation of the quinoline alkaloids.

Cyclopeptine dehydrogenase, as a pyridine nucleotide dependent dehydrogenase, is expected to react stereospecifically with position 4 of the nicotinamide moiety. To test this specificity NADH-[4-$^3H_1$] was prepared by incubating NAD$^+$-[4-^{3}H] in the presence of *S,R*-cyclopeptine and the enzyme (Fig. 1). After separation of the labelled NADH from unreacted NAD$^+$, by ion exchange chromatography, the stereochemical position of the tritium atom was determined utilizing alcohol dehydrogenase (E.C. 1.1.1.1) with acetaldehyde. The specific activity of the NAD$^+$-[4-^{3}H] formed by this reaction corresponded to the value calculated from the radioactivity of the originally used preparation. Because alcohol dehydrogenase is A-specific and removes the 4-pro*R*-hydrogen atom from NADH [12] it follows that this hydrogen atom must have been introduced by cyclopeptine dehydrogenase:



The same stereospecificity of cyclopeptine dehydrogenase was also shown by measurement of the reverse reactions. NADH-[4S-$^3H_1$] was prepared by reduction of NAD$^+$-[4-^{3}H] with ethanol and alcohol dehydrogenase. Incubation of the NADH-[4S-$^3H_1$] with dehydrocyclopeptine and cyclopeptine dehydrogenase gave unlabelled cyclopeptine in agreement with the conclusions drawn above.

EXPERIMENTAL

Cyclopeptine dehydrogenase was prepared from 6-day-old cultures of *Penicillium cyclopium* Westling strain SM 72 and purified about 70-fold by $(NH_4)_2SO_4$ and ethanol fractionation according to Aboutabl & Luckner [3]. For the synthesis of *S,R*-cyclopeptine and *S*-cyclopeptine see ref. 10, for the preparation of dehydrocyclopeptine see ref. 3. NAD$^+$-[4-^{3}H] (50 mCi/mmol) was obtained from the Radiochemical Centre Amersham (UK), DEAE-cellulose from Serva Heidelberg (BRD) and alcohol dehydrogenase (yeast) from VEB AWD

Dresden (DDR). Feeding of radioactive labelled cyclopeptine to cultures of *P. cyclopium* was performed as described by Framm *et al.* [2, table 1] by exchange of the culture medium. After 48 hr the buffer soln was filtered off and the alkaloids formed were separated and determined as given in Table 1.

Synthesis of cyclo-[anthranoyl-*N*-methyl-phenylalanyl]-3*S*-[10*R*-$^3H_1$]/3*R*-[1*S*-$^3H_1$] (Scheme 1). Benzaldehyde-[*CHO*-^3H] (**2**) [14] (2 mCi/mmol) was condensed [15] with *o*-nitrobenzoyl-sarcosine (**1**) to give *trans*-α-*N*-(*o*-nitrobenzoyl)-*N*-methyl-aminocinnamic acid-[3′-^3H] (**3**). 50 mg PtO_2 were reduced with H_2 to amorphous Pt and 6 mmoles of **3** were added to the Pt-suspension. The mixture was treated with H_2 at room temp. and normal pres until 18 mmol H_2 had been absorbed (reduction of the NO_2 group). Thereafter 8 mmol 2N HCl were added and the hydrogenation was continued until another 6 mmol H_2 had been consumed (reduction of the double bond). The methanolic soln was filtered and evaporated to dryness. It contained a mixture of *N*-(*o*-aminobenzoyl)-*N*-methyl-phenylalanine-2′-*S*-[3′*R*-$^3H_1$]/2′*R*-[3′*S*-$^3H_1$] (**4a** and **4b**) and of cyclo-[anthranoyl-*N*-methyl-phenylalanyl]-3*S*-[10*R*-$^3H_1$]/3*R*-[10*S*-$^3H_1$] (**5a** and **5b**). Yield 1.5 g (80% of the theoretical). For completion of the cyclization the residue was dissolved in a mixture of 10 ml HoAc, 5 ml H_2O and 5 drops of conc. HCl and heated 3 hr to 60–70°. After dilution with 1 vol. H_2O the mixture was adjusted to pH 8 with saturated Na_2CO_3 soln and extracted with $CHCl_3$. The washed organic phase was dried and evaporated. The residue, a yellow oil, was crystallized from EtOAc–petrol. Yield 0.57 g (40%), mp 158–160°, specific radioactivity 1.3 mCi/mmol.

Preparation of trans-α-N-(*o-nitrobenzoyl*-N-*methyl-aminocinnamic acid methyl ester. Trans*-α-*N*-(*o*-nitrobenzoyl)-*N*-methyl-aminocinnamic acid (5 mmol) synthesized according to ref. 15 was dissolved in MeOH and treated with an excess of CH_2N_2 in Et_2O. After evaporation the methylester was crystallized from Et_2O. Yield 1.53 g (90%), mp 122°.

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