Stereospecificity of the Enzymatic Dehydrogenation in the Biosynthesis of 3-Ethylidene-L-azetidine-2-carboxylic Acid from Isoleucine by *Streptomyces cacaoi*†

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ABSTRACT: (2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]Isoleucine and (2*S*,3*S*,4*R*)-[U-^{14}C,4-^{3}H]isoleucine have been prepared by stereospecific syntheses. The addition of these substrates to *Streptomyces cacaoi* led to the isolation of polyoxins from which 3-ethylidene-L-azetidine-2-carboxylic acid was isolated by hydrolysis. The *pro-R* hydrogen at C-4 of isoleucine was lost and the *pro-S* hydrogen was retained in the biosynthesis of 3-ethylidene-L-azetidine-2-carboxylic acid. These results indicate that the enzymatic dehydrogenation of isoleucine to form 3-ethylidene-L-azetidine-2-carboxylic acid involves the antiperiplanar elimination of the hydrogen at C-3 and the *pro-R* hydrogen at C-4.

The polyoxins are antifungal, naturally occurring peptidyl pyrimidine nucleoside antibiotics elaborated by *Streptomyces cacaoi* var. *asoensis* and *Streptomyces piomogenus* (Isono et al., 1965, 1969; Uramoto et al., 1978). The polyoxins, which are useful in the control of phytopathogenic fungi (Isono et al., 1967), are competitive inhibitors of chitin synthetase (Isono et al., 1969; Isono & Suzuki, 1968). Alkaline hydrolysis of polyoxins A, F, H, and K yields the three unusual α-L-amino acids polyoxin C (I), 3-ethylidene-L-azetidine-2-carboxylic acid (II), and 2-amino-2-deoxy-L-xylonic acid (III) (Figure 1). The biosynthesis of the pyrimidine chromophore, the aminouronic acid, and *trans-O*-carbamoyl-2-amino-2-deoxy-L-xylonic acid of the polyoxins has been reported (Isono et al., 1975, 1978; Isono & Suhadolnik, 1976; Funayama & Isono, 1975, 1976, 1977). Isoleucine has been reported to serve as the carbon skeleton for the biosynthesis of 3-ethylidene-L-azetidine-2-carboxylic acid (Figure 1, II). When [4,5-^{3}H]isoleucine was incorporated into 3-ethylidene-L-azetidine-2-carboxylic acid, four of the five hydrogens on C-4 and C-5 were retained (Isono et al., 1975). These results suggested that the reactions leading to the formation of the C-3,4 double bond proceeded via a mechanism by which at least one of the methylene hydrogens of C-4 was retained. Loss of the hydrogen atom at C-4 may occur by a stereospecific mechanism in which either the 4*R* or the 4*S* hydrogen of the prochiral methylene carbon of isoleucine is removed stereospecifically by a chiral enzyme. Dehydrogenation may occur first, followed by oxidation of the allylic methyl either to the alcohol or to the aldehyde, which would then cyclize to give an azetidine ring. Indeed, L-2-amino-3-(hydroxymethyl)-3-pentenoic acid and L-2-amino-3-formyl-3-pentenoic acid were obtained from *Bankera fuligineoalba* (Doyle & Lebenberg, 1968).

In this paper we report the stereospecific removal of the *pro-R* hydrogen on C-4 of isoleucine in the biosynthesis of the 3-ethylidene-L-azetidine-2-carboxylic acid moiety of polyoxins A, F, H, and K by *S. cacaoi*.

Materials and Methods

Synthesis of (2RS,3S,4S)-[2-^{14}C,4-^{3}H]Isoleucine. The stereospecific synthesis of this ^{14}C,^{3}H-labeled isoleucine has been described by Hill et al. (1980).

Synthesis of (2S,3S,4R)-[U-^{14}C,4-^{3}H]Isoleucine. The chemical synthesis of the ^{14}C,^{3}H-labeled isoleucine was as described by Crout et al. (1980).[1]

Incorporation of Doubly Labeled Isoleucine into 3-Ethylidene-L-azetidine-2-carboxylic Acid. The biosynthetic procedure is as described by Isono et al. (1975) except for the following minor changes. Fifteen 500-mL flasks containing 60 mL each of the culture medium were used. Sterile solutions of the radioactive isoleucine were added to the culture medium 77 h after inoculation, at the start of polyoxin production. After an additional 65 h, the polyoxin complex was isolated: yield, 600 mg (Isono et al., 1967). The polyoxin complex was hydrolyzed with 0.5 N NaOH on a steam bath for 2 h and 3-ethylidene-L-azetidine-2-carboxylic acid was isolated by using granulated charcoal chromatography (2.8 × 30 cm; solvent, water), instead of DE-52 chromatography: yield, 19.4 mg (Isono et al., 1975).

Results and Discussion

(2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]Isoleucine and (2*S*,3*S*,4*R*)-[U-^{14}C,4-^{3}H]isoleucine were added to polyoxin-producing cultures of *S. cacaoi*. 3-Ethylidene-L-azetidine-2-carboxylic acid was isolated, and the ratio of ^{3}H/^{14}C was determined. The data show that the enzymatic dehydrogenation occurring at C-3 and C-4 of isoleucine in the biosynthesis of the 3-ethylidene-L-azetidine-2-carboxylic acid moiety of the polyoxins results in the retention of the *pro-S* hydrogen but a complete loss of the *pro-R* hydrogen on C-4 (Table I). The ratio of ^{3}H/^{14}C in the (2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]isoleucine added to the cultures was 17, and the ratio of the ^{3}H/^{14}C in the 3-ethylidene-L-azetidine-2-carboxylic acid was 16. The ratio of the ^{3}H/^{14}C in the (2*S*,3*S*,4*R*)-[U-^{14}C,4-^{3}H]isoleucine which was resolved into the 2*S* isomer added to the cultures was 2.10, and the ratio of the ^{3}H/^{14}C of the 3-ethylidene-L-azetidine-2-carboxylic acid was 0.05. These data allow us to conclude that the conversion of isoleucine into 3-ethylidene-L-azeti-

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[1] This paper describes the synthesis of the deuterio-labeled analogue of [4(*R*)-^{3}H]isoleucine.

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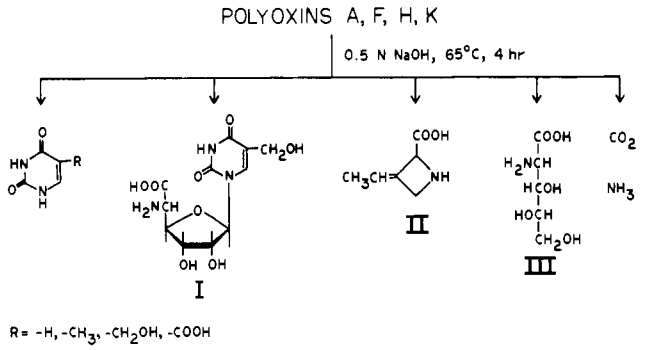

FIGURE 1: Alkaline hydrolysis products of polyoxins A, F, H, and K (Isono et al., 1975).

Table I: Incorporation of (2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]- and (2*S*,3*S*,4*R*)-[U-^{14}C,4-^{3}H]Isoleucine into 3-Ethylidene-L-azetidine-2-carboxylic Acid

isoleucine added[a]				3-ethylidene-L-azetidine-2-carboxylic acid isolated		
	sp act. (μCi/μmol)			sp act. (nCi/μmol)		
	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C
(2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]	0.074	1.26	17.0	0.159	2.55	16.0
(2*S*,3*S*,4*R*)-[U-^{14}C, 4-^{3}H]	0.150	0.316	2.10	0.18	0.009	0.05

[a] A total of 53.5 μmol (6.8 mg) of (2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]isoleucine and 19.9 μmol (2.61 mg) of (2*S*,3*S*,4*R*)-[U-^{14}C,4-^{3}H]isoleucine was added in the given specific activities into fifteen 500-mL flasks of 77-h cultures of *S. cacaoi*. Other procedures are the same as reported previously (Isono et al., 1975).

dine-2-carboxylic acid involves overall antiperiplanar elimination of the hydrogen at C-3 and the *pro-R* hydrogen at C-4 (Figure 2).

Examples of either stereospecific or nonstereospecific enzymatic removal of methylene hydrogens have been reported. For example, the biosynthesis of tiglic acid by *Datura innoxia* proceeds via the enzymatic dehydrogenation of the 4*R* hydrogen of [4*S*-^{3}H]isoleucine which involves antiperiplanar elimination of the hydrogen at C-2 and the *pro-R* hydrogen on C-3 at (2*S*)-2-methylbutanoic acid (Hill et al., 1980). Although the introduction of the C-3,4 double bond into 3-ethylidene-L-azetidine-2-carboxylic acid may not occur by direct dehydrogenation, it is pertinent to note that enzymatic reactions proceeding by both syn and anti dehydrogenation are known (Rose, 1972). An example of a syn-periplanar elimination (cis elimination) is the report by Luckner and co-workers (Aboutabl et al., 1976). They showed the 10-*pro-S* hydrogen of the benzodiazepine alkaloid, (3*R*)-[10(*S*)-^{3}H]-cyclopeptine, was removed by cyclopeptine dehydrogenase. The dehydrogenase removed the two hydrogen atoms at C-3 and C-10 of cyclopeptine to form *trans*-dehydrocyclopeptine. The hydride ion removed from cyclopeptine was transferred to the 4-*pro-R* position of NAD. Thus, cyclopeptine dehydrogenase belongs to the A-specific dehydrogenases. It is also worth noting that during the formation of mycelianamide from (2*S*)-[3(*R*)-^{3}H]tyrosine by *Penicillium griseofulvum*, the elimination of the 2*S* and 3*S* hydrogen atoms would be expected to proceed via a syn-periplanar elimination (Kirby & Narayanaswami, 1973).

Several acyl-CoA dehydrogenases have been reported to act in a totally stereospecific manner. Biellmann & Hirth (1970a,b) as well as Bucklers et al. (1970) reported that the

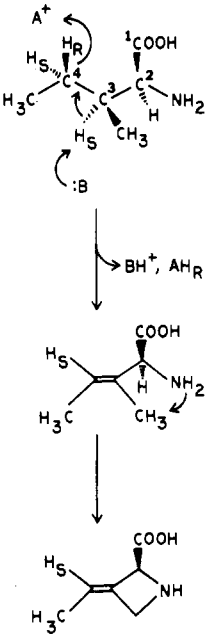

FIGURE 2: Antiperiplanar elimination of the *pro-R* hydrogen at C-4 of isoleucine in the biosynthesis of 3-ethylidene-L-azetidine-2-carboxylic acid by *S. cacaoi*. A^{+}, electron acceptor; :B, electron donor.

dehydrogenation of butanoyl-CoA by butyric acid dehydrogenase of hog liver specifically removes the *pro-R* hydrogens at C-2 and C-3. With isobutyryl-CoA dehydrogenase, the α- and β-hydrogens are specifically removed from the *pro-S* methyl group (Amster & Tanaka, 1980). Stereospecific and nonstereospecific removal of the *pro-R* and *pro-S* hydrogens in other types of enzyme reactions have been reported. For example, Floss and co-workers (Tsai et al., 1979) reported that indole-3-alkane α-hydroxylase removes stereospecifically the *pro-S* hydrogen at C-2 of the side chain of tryptamine. Bryant & Benkovic (1979) and Burgers & Eckstein (1978) reported that snake venom phosphodiesterase catalyzes the hydrolysis of the *S* enantiomer of *O*-(*p*-nitrophenyl) phenylphosphothionate. However, in the case of the physiological entry of C-3 of serine into the C-1 pool, Tatum et al. (1977) reported that the transfer of the prochiral hydroxymethyl group of either (3*R*)- or (3*S*)-serine to tetrahydrofolate by serine transhydroxymethylase procedes via a nonenzymatic racemization of the prochiral methylene center. Diziol et al. (1980) reported that ethanolamine ammonia-lyase can catalyze the deamination of two enantiomeric substrates. More recently, Graves et al. (1980) reported that in the deamination of 2-aminopropanol the (*S*)-2-aminopropanol is a better substrate than the *R* enantiomer. These results are unusual, because most enzymes are highly specific with respect to the stereochemical configuration of their substrates. Their data suggest that a hydrogen transfer step is rate limiting with 2-aminopropanol as the substrate.

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Glutamate as the Common Precursor for the Aglycon of the Naturally Occurring C-Nucleoside Antibiotics†

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ABSTRACT: Pyrazofurin is one of four naturally occurring C-nucleoside antibiotics; it is elaborated by *Streptomyces candidus*. The biosynthesis of the pyrazole ring of pyrazofurin has been studied by using ^{13}C- and ^{14}C-labeled acetate. Carbon-13 incorporation into pyrazofurin was observed by proton-decoupled ^{13}C Fourier transform NMR spectroscopy. The incorporation of ^{14}C from [1-^{14}C]acetate was 0.7%. The enrichment of carbons 3, 4, and 5 of pyrazofurin from [2-^{13}C]acetate by *S. candidus* confirms earlier findings that acetate is converted to glutamate by the combined action of the Krebs cycle and malic enzyme [Elstner, E. F., Suhadolnik, R. J., & Allerhand, A. (1973) *J. Biol. Chem. 248*, 5385]. Malic enzyme will give rise to [1,2-^{13}C]acetate from [2-^{13}C]acetate. The [1,2-^{13}C]acetate is then converted to glutamate labeled with ^{13}C in carbons 2–5. The ^{13}C incorporation data indicate that carbons 1, 2, 3, and 4, but not 5, of glutamate serve as the four-carbon donor for the carboxamide carbon, C-5, C-4, and C-3, respectively, of the pyrazole ring of pyrazofurin.

Four naturally occurring C-nucleoside antibiotics have been isolated from the *Streptomyces*. They are showdomycin, oxazinomycin (minimycin), formycin, and pyrazofurin [see Figure 2 for structures; for reviews, see Bloch (1978) and Suhadolnik (1979, 1981)]. These C-nucleoside antibiotics inhibit RNA and DNA synthesis in bacteria, tumor cells, and viruses without affecting the nucleotide, RNA, and DNA processes in the *Streptomyces*-producing organisms. Pyrazofurin is of special interest because it represses replication of vaccinia, herpes simplex, measles, rhino, and influenza viruses in cell culture, inhibits the formation of vaccinia tail lesions in mice, and is in current testing for the treatment of breast cancer (Descamps & De Clercq, 1978; Gutowski et al., 1975; Cadman et al., 1978a; Hill & Whelan, 1980). Pyrazofurin, after conversion to its 5′-phosphate, competitively inhibits orotidylate decarboxylase (Sweeney et al., 1973; Cadman et al., 1978b; Dix et al., 1979). The chemical syntheses of showdomycin have been reported by Kalvoda (1976) and Trummlitz & Moffatt (1973), oxazinomycin by De Bernardo & Weigele (1977), formycin by Kalvoda (1976, 1978) and Buchanan et al. (1980a), and pyrazofurin by De Bernardo & Weigele (1976) and Buchanan et al. (1980b).

The finding that UMP serves as the precursor for pseudouridine 5′-monophosphate in tRNA (Uematsu & Suhadolnik, 1973; Cortese et al., 1974), together with earlier reports from this and other laboratories on the biosynthesis of the *N*-ribosyl antibiotics in which the purine ring serves as the precursor for

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