3,4-Dimethylindole-2-carboxylate and 4-(1-Hydroxyethyl)quinoline-2-carboxylate Activating Enzymes from the Nosiheptide and Thiostrepton Producers, *Streptomyces actuosus* and *Streptomyces laurentii*

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Highly specific enzymes which activate 3,4-dimethylindole-2-carboxylate (DMI) and 4-(1-hydroxyethyl)quinoline-2-carboxylate (HEQ) to their acyl adenylates have been detected in cell-free extracts of the nosiheptide and thiostrepton producers, *S. actuosus* and *S. laurentii*, by an ATP/PP_i exchange assay.

Nosiheptide **1**[1] and thiostrepton **2**[2] (Fig. 1) are structurally related members of a class of sulfur-rich peptide antibiotics which are produced primarily by bacteria of the genus *Streptomyces*. These thiopeptide antibiotics inhibit protein synthesis in Gram-positive bacteria by forming a complex with the 23S rRNA and ribosomal protein L11.[3–7] Thiopeptide antiobiotics are used commercially as feed additives in agriculture, and in veterinary applications.[8] Resistance to these antibiotics is conferred upon the producing streptomycetes by a methytransferase that methylates the 23S rRNA.[6,9–12]

Both **1** and **2** are formed from amino acids which are assembled into highly modified peptides consisting of a large macrocycle containing thiazole or thiazoline rings and a side chain with a variable number of dehydroalanine residues.[13,14] In **2**, a second loop links the amino-terminal isoleucine to a threonine *via* a modified quinaldic acid (the 'quinaldic acid' moiety). Similarly, in **1**, a smaller second loop is formed by linking a cysteine to the γ-carboxy group of hydroxyglutamic acid *via* a modified indole-2-carboxylic acid (the 'indolic acid' moiety). Labelling studies have shown that both the quinaldic and the indolic acid moieties are biosynthesized from tryptophan.[13–15] It has been proposed that tryptophan is converted into the free intermediates 3,4-dimethylindole-2-carboxylate (DMI **3**) in *S. actuosus* and 4-(1-hydroxyethyl)-quinoline-2-carboxylate (HEQ **4**) in *S. laurentii* before attachment to the thiopeptide backbone.[15] ^3H Labelled DMI or ^{13}C-labelled HEQ are efficiently and specifically incorporated into **1** and **2**, respectively.[15]

There are many natural products which contain modified carboxylic acids in ester or amide linkages. The producing organisms usually also contain enzymes that activate the carboxy groups, most commonly as acyl adenylates.[16–21] In this paper, we present evidence demonstrating that DMI and HEQ are activated as acyl adenylates (**5** and **6**) before incorporation into the respective antibiotics, **1** and **2**.

The conversion of carboxylic acids to their acyl adenylates requires ATP, and produces inorganic pyrophosphate (Scheme 1, i and ii). Since the reaction is reversible, activity can be monitored by measuring the incorporation of ^{32}P into ATP when the substrate, ATP, Mg^{2+} and [^{32}P]-PPi are incubated with the activating enzyme.[16,22,23] The enzymes from *S. laurentii* (Calbiochem) and *S. actuosus* ATCC 25421 were enriched 5–10-fold by passing clarified cell-free extracts over DEAE-cellulose, and then fractionating with ammonium sulfate.[16] The partially purified enzymes catalysed and ATP–

PPi exchange reaction that was dependent on either DMI (*S. actuosus*), or HEQ (*S. laurentii*), Mg^{2+}, and ATP (Table 1). HEQ-dependent exchange activity was not observed in extracts of *S. actuosus* nor was DMI-dependent exchange exchange activity observed in *S. laurentii* extracts.

With both enzymes, all substrates tested showed simple saturation kinetics. Apparent† K_m values for the substrates were estimated from Lineweaver–Burk plots derived from the extent of exchange measured at various concentrations of either DMI or HEQ at saturating fixed concentrations of ATP. Similarly, apparent K_m values for ATP, in each system, were determined at fixed, saturating concentrations of either DMI or HEQ (Table 1). Apparent K_m values of 4 μmol l^{-1} for DMI and 3.0 mmol l^{-1} for ATP were determined with the partially purified *S. actuosus* enzyme. The *S. actuosus* enzyme was specific for DMI and its analogue 3-methylindole-2-carboxylate (3-MI). Both 3-MI and DMI can be incorporated into **1**.[15] Apparent K_m values of 3 μmol l^{-1} for racemic HEQ and 0.3 mmol l^{-1} for ATP were measured with the partially purified *S. laurentii* enzyme. Both enantiomers of HEQ were separately synthesized (85% enantiomeric excess),[24] and tested as substrates. The concentration of (*R*)-HEQ which gave 50% of the maximal exchange rate was found to be ten-fold higher than the concentration of (*S*)-HEQ which gave 50% of its maximal exchange rate; the small amount of activity observed with the (*R*)-enantiomer is probably due to contamination with the (*S*)-enantiomer (*ca.* 7.5%). These data show that the *S. laurentii* activating enzyme is highly selective for the stereoisomer of HEQ found in **2**. The substrate selectivity was further examined with a series of HEQ analogue differing

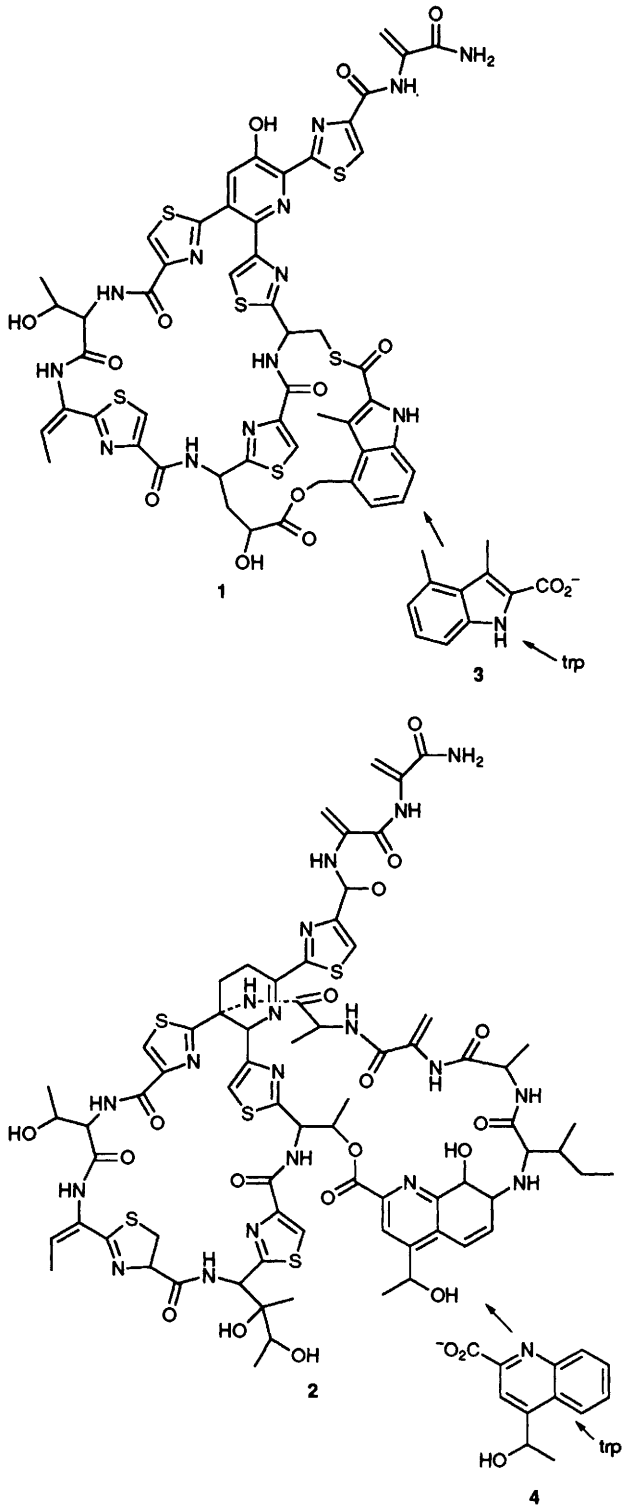
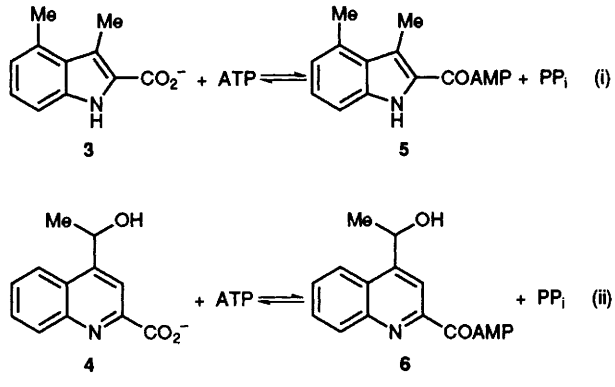


Fig. 1 Structures of nosiheptide **1** and thiostrepton **2**. The proposed intermediates (**3** and **4**) arising from tryptophan are shown.

† Only apparent K_m and V_{max} values can be measured because the initial concentration of the true substrate (the acyl adenylate) is zero at the start of the incubation, and the products of the reverse reaction (HEQ or DMI and ATP) are present in excess. The exchange reaction was linear to 30 min with the *S. laurentii* enzyme, and was not determined for the *S. actuosus* enzyme.



Scheme 1

Table 1 ATP/PPi exchange activity in partially-purified extracts of *S. actuosus* and *S. laurentii*[a]

Sample	[nmol PPi exchanged]	
	S. actuosus	*S. laurentii*
With DMI	0.16	0.06
With HEQ	0.05	0.83
Without enzyme	0.04	0.04
Without ATP	0.02	0.06
Without substrate	0.06	0.07
With 10 mmol l^{-1} EDTA	0.04	0.04

[a] Exchange activity was determined by incubating 10 μl (*S. laurentii*) or 50 μl (*S. actuosus*) of enzyme solution for 10 min, at room temperature, with 10 mmol l^{-1} ATP, 5 mmol l^{-1} $MgCl_2$, 5 nmol PPi (5 × 10^5 CPM [^{32}P]PPi), 5 mmol l^{-1} substrate (DMI or HEQ), and 50 mmol l^{-1} KPi buffer, pH 6.8, in a volume of 100 μl. 5 μl of the reaction mixture were removed and the radioactivity was measured (initial CPM). The incubations were stopped by the addition of activated charcoal [50 μl, 5.0% (w/v) suspension in 10 mmol l^{-1} ethylenediaminetetracetate, EDTA, pH 8.0]. Free PPi was removed by washing the charcoal pellet with water. The washed pellet was resuspended in 100 μl of water and 50 μl of the suspension was counted (final CPM). The amount PPi exchanged was calculated by dividing the final CPM (multiplied by 2) by the initial CPM (multiplied by 20) and multiplying the resulting value by 5 nmol (amount of PPi added). As a control, the radioactive ATP was eluted with aqueous pyridine [100 μl, 10% (v/v)], and the eluate (2 μl) was analysed by TLC on PEI-cellulose. The TLC data were compared with the counting data to determine the background binding of PPi to the charcoal (without extract). The data reported are the average of duplicate measurements.

in the substitution at C-4. Only one analogue, 4-hydroxymethylquinoline-2-carboxylate (HMQ), showed activity. When compared to racemic HEQ the relative apparent V_{max} value of HMQ was *ca.* 0.60. The apparent K_m value for HMQ was estimated to be 80 μmol. Other analogues, 4-acetylquinoline-2-carboxylate, 4-methylquinoline-2-carboxylate and quinoxaline-2-carboxylate and quinoxaline-2-carboxylate, were not active. The methyl ester of HEQ was found to be a competitive inhibitor of the ATP–PPi exchange reaction, catalysed by the *S. laurentii* extract, with an apparent K_i of 28 μmol l^{-1}. The native molecular weight of the HEQ activating enzyme was estimated by gel permeation chromatography to be $(47 \pm 4) \times 10^3$. This value is in good agreement with the molecular weights determined for other carboxylate-activating enzymes involved in the biosynthesis of peptide-based secondary metabolites.[16–21]

From these data it is concluded that *S. actuosus* and *S. laurentii* contain enzymes that activate DMI (*S. actuosus*) and HEQ (*S. laurentii*) to their corresponding acyl adenylates which is believed to be necessary for their attachment to the peptide backbones of **1** and **2**, respectively. The ultimate proof that formation of an acyl adenylate is a requirement for attachment will require inactivation of the genes for these enzymes and subsequent demonstration that neither DMI nor HEQ is incorporated into the antibiotics.

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