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Studies on the Biosynthesis of Thiostrepton: 4-(1-Hydroxyethyl)quinoline-2-carboxylate as a Free Intermediate on the Pathway to the Quinaldic Acid Moiety

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Abstract—Specifically ^{13}C-labeled quinoline-2-carboxylate derivatives were synthesized from quinoline and used to study the biosynthesis of thiostrepton in a strain of *Streptomyces laurentii*. ^{13}C NMR analysis of thiostrepton recovered after feeding methyl (*RS*)-[11-^{13}C]-4-(1-hydroxyethyl)quinoline-2-carboxylate or methyl [11-^{13}C]-4-acetylquinoline-2-carboxylate showed conclusively that these compounds are specifically and efficiently incorporated into thiostrepton. Both compounds were also detected in cultures of the producing organism by isotope dilution analysis. The significance of the relative endogenous concentrations of the two compounds and of the relative extent of the incorporation of exogenously added labeled material into thiostrepton are discussed in terms of the biosynthetic pathway linking tryptophan and 4-(1-hydroxyethyl)quinoline-2-carboxylate in *S. laurentii*. A highly specific enzyme activity was detected in cell-free extracts of *S. laurentii* that was capable of adenylating (12*S*)-4-(1-hydroxyethyl)quinoline-2-carboxylic acid. Partial purification of the enzyme was achieved. The enzyme was found to be specific for the enantiomer of the substrate which has the same absolute configuration as found in the natural antibiotic structure. The presence of one specific enzyme catalysing the adenylation process in *S. laurentii* was shown by photoaffinity labeling with [α-^{32}P]-8-azido-ATP and subsequent SDS PAGE analysis of the labeled products. The native molecular weight of the active enzyme, determined by gel permeation chromatography, was found to be approximately 47 kDa, compared with a denatured weight of 50 kDa estimated for the photoaffinity-labeled protein. The enzyme is thus probably monomeric. Copyright © 1996 Elsevier Science Ltd

Introduction

Thiostrepton[1] (**1**) is one of the best characterized members of a class of sulfur-rich peptide antibiotics that also includes nosiheptide,[2] the siomycins,[3] the micrococcins,[4] and the thiopeptins.[5] Thiostrepton is produced by *Streptomyces azureus* ATCC 14921, *S. hawaiiensis* ATCC 12236, and by *S. laurentii* ATCC 31255. It is used as a topical veterinary antibiotic[6] and as a selection tool in recombinant DNA experimentation. Low solubility and poor bioavailability have precluded its introduction into human medicine. Compound **1** is active against Gram positive bacteria; it inhibits protein biosynthesis by binding to the 23*S* ribosomal RNA and ribosomal protein L11, thus blocking the GTPase-dependent activities of the 50S ribosomal subunit.[7] Resistance to **1** is conferred by a methylation of adenosine-1067 of the 23*S* ribosomal RNA.[8]

The structure of **1** was elucidated by X-ray crystallography[9] in combination with degradative and spectroscopic techniques;[10,11] some of the ^1H and ^{13}C NMR assignments were later revised.[12] Feeding experiments with ^{13}C-labeled putative precursors showed that all of the structural elements of **1** are biosynthetically derived from various amino acids (Fig. 1).[13,14] The quinaldic acid moiety (Fig. 1) of **1** is derived almost entirely from tryptophan; only the methyl group comes from methionine. When D,L-2-methyl-[3′-^{13}C]tryptophan was fed to a culture of *S. laurentii* the resulting thiostrepton was shown by ^{13}C NMR spectroscopy to carry about 40% ^{13}C at C-3 of the quinaldic acid moiety.[13,14] 2-Methyltryptophan can be detected in extracts of *S. laurentii*, and an enzyme activity catalyzing the methylation of tryptophan at C-2 of the indole ring has been partially characterized.[15] Furthermore, when L-[*indole*-^{15}N,1′,2′-$^{13}C_2$]tryptophan was fed to a culture of *S. laurentii*, the **1** isolated was ^{13}C-enriched both at C-2 and the carboxyl group of the quinaldic acid moiety. The ^{13}C NMR signal assigned to C-2 showed a 2-bond coupling to ^{15}N,[14] indicating that 2-methyltryptophan is converted into the quinaldic acid moiety by an intramolecular rearrangement connecting the indole-N to the C-2′ of 2-methyltryptophan. It was proposed[13,14] that this rearrangement leads to 4-acetylquinoline-2-carboxylic acid (**3**), which is reduced to 4-(1-hydroxyethyl)quinoline-2-carboxylic acid (**4**) and incorporated into **1** via the activated intermediates **5** and **2**[16] (Fig. 2).

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In the present paper we present the results of further experiments designed to test these ideas.

Results

Synthesis

For the present studies a number of quinoline derivatives were required, especially the enantiomerically pure stereoisomers of the alcohol **4**, and specifically ^{13}C-labeled forms of ketone **3** and alcohol **4**. The stereoisomers of **4** were prepared as shown in Figure 3. Quinoline-2-carboxylic acid (**6**) was obtained from commercial sources or prepared from quinoline on a larger scale.[17,18] Methylation of **6** proceeded cleanly and in high yield using $SOCl_2$ in MeOH on scales from 0.1 to 30 g of starting material. Introduction of an acetyl group at the 4-position of the quinoline nucleus was achieved by a modification of the homolytic aromatic substitution chemistry developed by Minisci et al.[19] An $FeSO_4$–H_2O_2 system was used to generate MeCO· radicals from acetaldehyde, which under acidic conditions add to the protonated quinoline ring specifically at C-4, since the 2-position is blocked by the carbomethoxy group. Nearly quantitative yields were obtained when acetaldehyde was present in excess (used as the solvent). Reduction of the ketone **8** with $NaBH_4$ in methanol affords the racemic alcohol **9** in nearly quantitative yield. Substitution of other alcohols for methanol as solvent led to side reactions.

An enantiomerically enriched sample of the alcohol **9** was synthesized by hydrolyzing the racemic butyrate derivative **10** with *Candida cylindracea* lipase (CCL). Enantiomeric excess values (ee) were measured by derivatizing the alcohol with (1*S*)-camphanyl chloride and then determining the diastereomeric excess (de) by ^1H NMR. Although the derivatization reaction suffers from some kinetic resolution it is not difficult to achieve near quantitative conversions of the alcohol samples. An intriguing temperature dependence for the stereoselectivity of the *Candida cylindracea* lipase was serendipitously noted. At room temperature the alcohol product can be obtained in approximately 40% ee at 10% overall conversion. This corresponds to an E-value[20] of approximately 1.2–1.5. At 50 °C the stereoselectivity is much higher. For example, one large-scale preparation gave the alcohol in >85% ee at

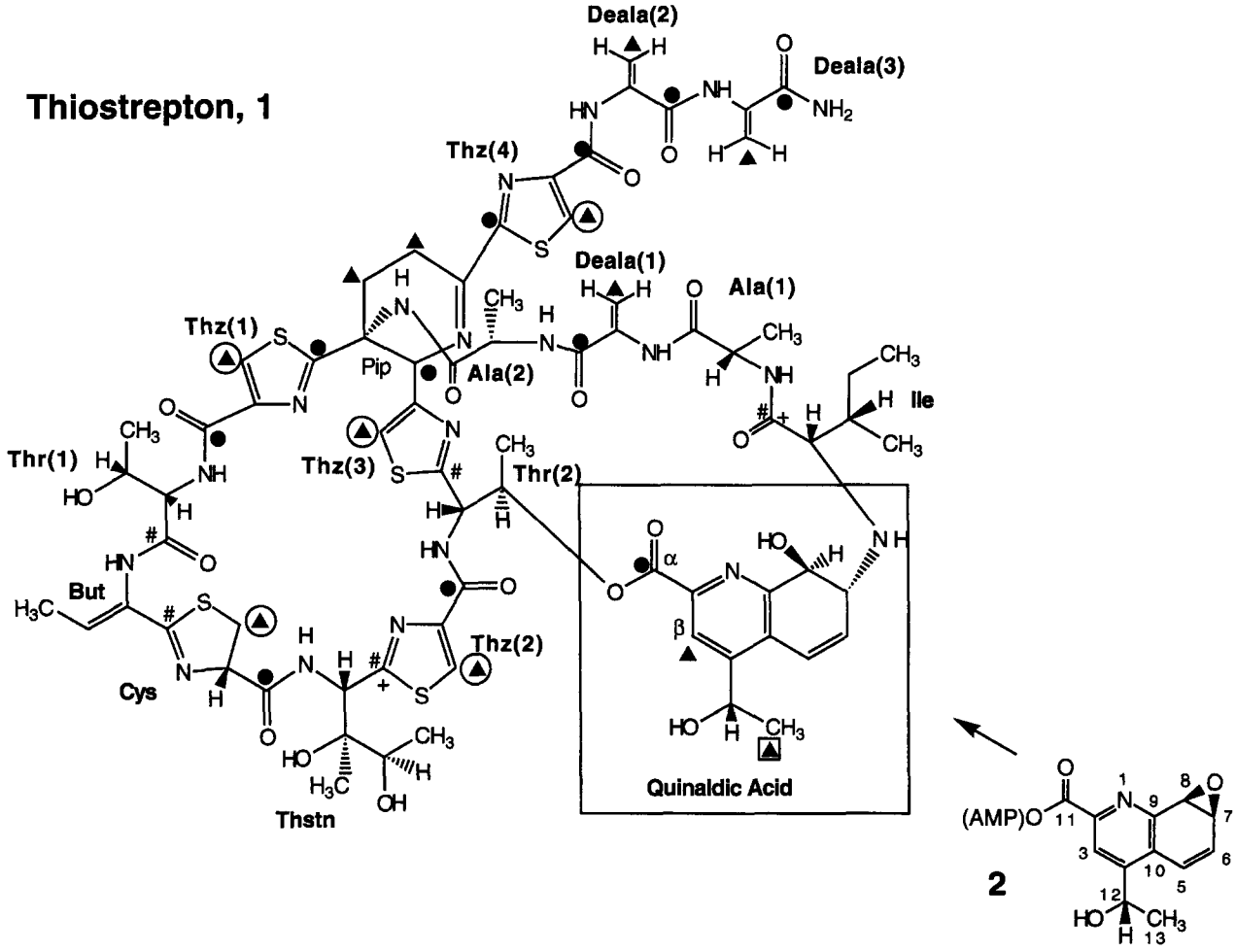

Figure 1. Biosynthetic origin of thiostrepton: Positions of label from D,L-[3-^{13}C]cysteine (O), D,L-[1-^{13}C]serine (●), D,L-[3-^{13}C]serine (▲), D,L-[1-^{13}C]isoleucine (+), D,L-[1-^{13}C]threonine (#), L-[methyl-^{13}C]methionine (□), L-[1′-^{13}C]tryptophan (α), and D,L-2-methyl-[3′-^{13}C]tryptophan (β).

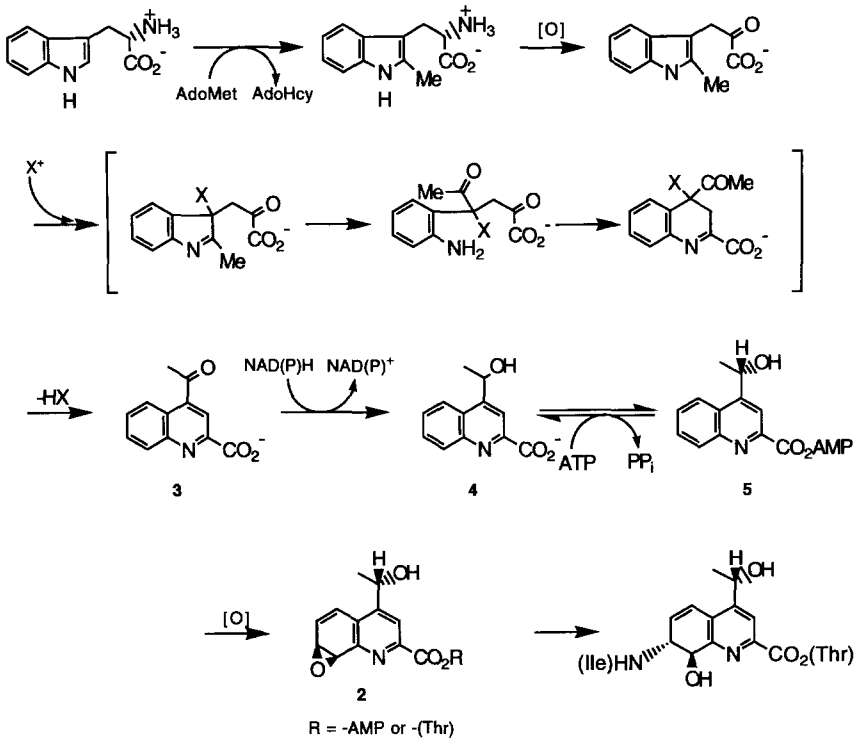

Figure 2. Proposed pathway for the conversion of tryptophan into the quinaldic acid moiety of thiostrepton (AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine).

17.4% conversion, corresponding to an E-value of approximately 15. Although recrystallization of the camphanate derivative of *rac*-**9** did not resolve the diastereomers, crystallization of the diastereomerically enriched camphanate afforded the diastereomerically pure derivative (de >98% by ^{1}H NMR, no peaks corresponding to the minor diastereomer were seen). The lipase procedure proved to be both reproducable and reliable.

The absolute configuration of **9** recovered from the CCL hydrolysis was determined by ^{1}H NMR analysis of its Mosher ester, **14**, by the method of Ohtani et al.[21] From the relative chemical shift values for resonances assigned to protons near the carbinol center of both the major and minor diastereomers of **14**, the absolute configuration of the alcohol recovered from the lipase incubations was determined to be 12*R*. This agrees with precedent for other lipase reactions catalyzed by CCL.[22] The benzoate derivative, **11**, of (12*S*)-**9** could be readily obtained by the Mitsunobu procedure,[23] and the acids (*S*)-**4** and (*R*)-**4** were then generated either by base hydrolysis of (12*S*)-**11** or the alcohol (12*R*)-**9**, respectively, or in enantiomerically pure form, by hydrolysis of the (1*S*)-camphanate esters.

In an attempt to improve the stereoselectivity of the hydrolysis by changing the ester moiety, the CCL-catalyzed hydrolysis of the acetate **12** and the caproate **13** were studied. It was found that the rate of hydrolysis decreased in the order **13**>**10**>**12**, with the acetate being essentially unreactive. Concomitant with the ease of hydrolysis of the caproate, the enantiomeric excess of the recovered alcohol was very low.

Additional analogues of **4**, namely 4-methyl- and 4-hydroxyquinoline-2-carboxylic acid, were synthesized from 4-methyl- and 4-hydroxyquinoline, respectively, using Popp's modification[17] of Reissert-type chemistry.[18] Treatment of **7** with $FeSO_4$–H_2O_2 in methanol gave the 4-hydroxymethyl analogue, **15** (Fig. 3). Hydrolysis of **15** in base provided the corresponding acid **16**.

Synthesis of labeled compounds

For the preparation of [11-^{13}C]-**8** and *rac*-[11-^{13}C]-**9**, [11-^{13}C]-**7** was synthesized from quinoline as shown in Figure 4. The Reissert reaction was slightly modified so that the labeled KCN, rather than quinoline, was present as the limiting reagent. The ^{13}C-labeled Reissert compound (**17**) was obtained in 75% yield as white crystals. Hydrolysis of **17** was carried out using the AcOH/HBr system of Davis[24] to give the hydrobromide salt of the labeled acid, **18**.

In larger scale syntheses of unlabeled compounds the equivalent of the hydrobromide **18** could readily be converted to the free acid **6** prior to methylation. The neutralization, however, gave poor recoveries of material when attempted on a small scale. The hydrobromide **18** was, therefore, converted directly to its methyl ester [11-^{13}C]-**7** by neutralization with one equivalent of NaOH followed by methylation with $SOCl_2$/MeOH in a 'one-pot' procedure. Direct methylation of unlabeled **18** with either $SOCl_2$/MeOH, H^+/MeOH, or $(MeO)_2SO_2/K_2CO_3$ gave inadequate yields of **7**. The further conversion of [11-^{13}C]-**7** into [11-^{13}C]-**8** and [11-^{13}C]-**9** followed the procedures used with the corresponding unlabeled compounds.

Feeding experiments

Feeding experiments were undertaken to evaluate the roles of the 4-acetylquinoline-2-carboxylic acid **3** and the corresponding hydroxyethyl compound **4** in the biosynthesis of the quinaldic acid moiety of **1**. Since it was suspected that the free acids, being amphoteric, might not penetrate the cell membranes of the organism very well, the methyl esters were used instead, relying on intracellular esterases to cleave these 'prodrugs' into the active species. [11-^{13}C]-**8** (100 mg) and *rac*-[11-^{13}C]-**9** (116.5 mg) were each added to five cultures of *S. laurentii*, which were fermented for 3 days. The yields of isolated **1** (18 and 11.2 mg) in both cases were substantially lower than those obtained from comparable cultures to which no exogenous compounds had been added. To ascertain isotopic purity, the isolated samples were diluted with a known amount of authentic **1**, repurified, and then analyzed by ^{13}C NMR to determine the degree of ^{13}C-incorporation (Fig. 5). The **1** from both feeding experiments showed significant incorporation of the ^{13}C-label. The chemical shift of the enriched signal in both spectra corresponds to the carboxyl carbon (C-11) of the quinaldic acid moiety of **1**.[12] In both cases only one ^{13}C-signal showed significant enrichment, $29 \pm 10\%$ and $81 \pm 10\%$, respectively, in the [11-^{13}C]-**8** and [11-^{13}C]-**9** feeding experiments.

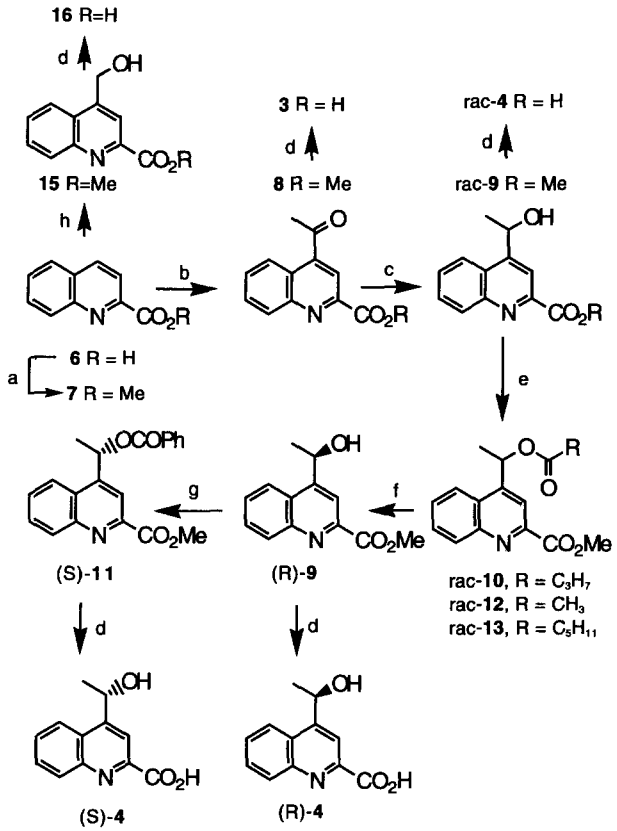

Figure 3. Synthesis of 4-hydroxymethylquinoline-2-carboxylate and of optically active samples of 4-(1-hydroxyethyl)quinoline-2-carboxylate **4**: (a) $SOCl_2$, MeOH, Δ; (b) CH_3CHO, H_2O_2, $FeSO_4$, TFA; (c) $NaBH_4$, MeOH; (d) NaOH–THF/H_2O; (e) RCOCl, pyridine, CH_2Cl_2; (f) *Candida cylindracea* lipase, pH 7, DMSO–H_2O; (g) DEAD, $PhCO_2H$, Ph_3P, CH_2Cl_2; (h) TFA, H_2O_2, $FeSO_4$, MeOH, Δ.

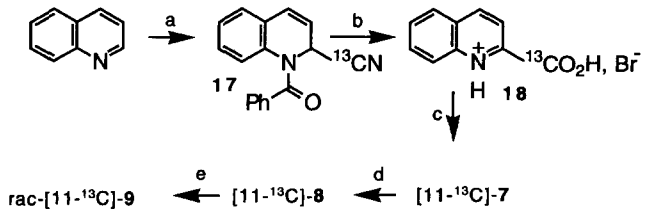

Figure 4. Synthesis of labeled quinoline derivatives: (a) PhCOCl, $K_{13}CN$, CH_2Cl_2–H_2O; (b) HOAc, HBr, Δ; (c) NaOH then $SOCl_2$, MeOH, Δ; (d) CH_3CHO, H_2O_2, $FeSO_4$, TFA; (e) $NaBH_4$, MeOH.

Isotope dilution experiments

To determine whether **3** and **4** are present in the fermentations, and at what concentrations, known amounts of [11-^{13}C]-**3** and *rac*-[11-^{13}C]-**4** were added to separate cultures of *S. laurentii* to act as internal standards. Immediately following the addition, the cells were broken and the resulting broth samples were acidified, extracted with *n*-BuOH and the extracts treated with diazomethane. The methyl esters **8** and **9** were purified by preparative TLC and analyzed by GC-MS for a decrease in ^{13}C content due to the dilution with unlabeled **3** and **4** present in the fermentations. It was found that at 44 h after inoculation, **3** and **4** were present at 47 ± 2 and 254 ± 2 mM, respectively, in production cultures of *S. laurentii*.

Carboxylate activation

The incorporation of **4** into the quinaldic acid moiety of **1** presumably requires activation of both the carboxyl group and the aromatic ring to set the stage for the two bond formations to the peptide core (Fig. 2). The possibility that the carboxyl group of **4** is activated first, as an acyl adenylate as in analogous cases,[25–27] was tested by looking for an enzyme activity catalyzing such a reaction using an in vitro assay.[25,28] Cell-free extracts obtained from 3-day old production cultures of *S. laurentii* were incubated with *rac*-**4**, Mg^{2+}, ATP, and ^{32}P-labeled pyrophosphate (PP_i). Since the adenylation reaction is reversible (Fig. 2) an enzyme capable of catalyzing this reaction will catalyze the incorporation of ^{32}P-label from ^{32}P-PP_i into ATP. ATP was recovered from the incubation mixture by adsorption on activated charcoal and excess PP_i was removed by washing the charcoal with water. ATP could be eluted from the charcoal with aqueous pyridine. The extent of incorporation of ^{32}P into ATP was measured by scintillation counting of the aqueous pyridine washings or, more simply, by direct counting of the dispersed charcoal pellet. To show that the radioactivity was present specifically in the ATP, the pyridine eluates were analyzed by polyethyleneimine-cellulose (PEI-cellulose) TLC and autoradiography. No incorporation of ^{32}P into ATP was seen when either *rac*-**4** or ATP were omitted from the incubation, when

the cell-free extract was replaced by bovine serum albumin, or when excess EDTA was added to the incubation mixture. When all the necessary components are present, however, a significant proportion of the ^{32}P is incorporated into ATP. Under the usual assay conditions the rate of ^{32}P incorporation into ATP was linear for at least 30 min. All subsequent assays were conducted within this linear time period. The enzyme activity was enriched about 5- to 10-fold over the crude extract by PEI- and DEAE-cellulose treatment, 65% ammonium sulfate precipitation and desalting with Sephadex G-25.

Kinetic parameters and substrate specificity

Various kinetic parameters were determined for the adenylyl transferase and a variety of substrates. Apparent[29] K_m values for *rac*-**4** and (12*S*)-**4** were determined from Lineweaver–Burk plots[30] of the extent of

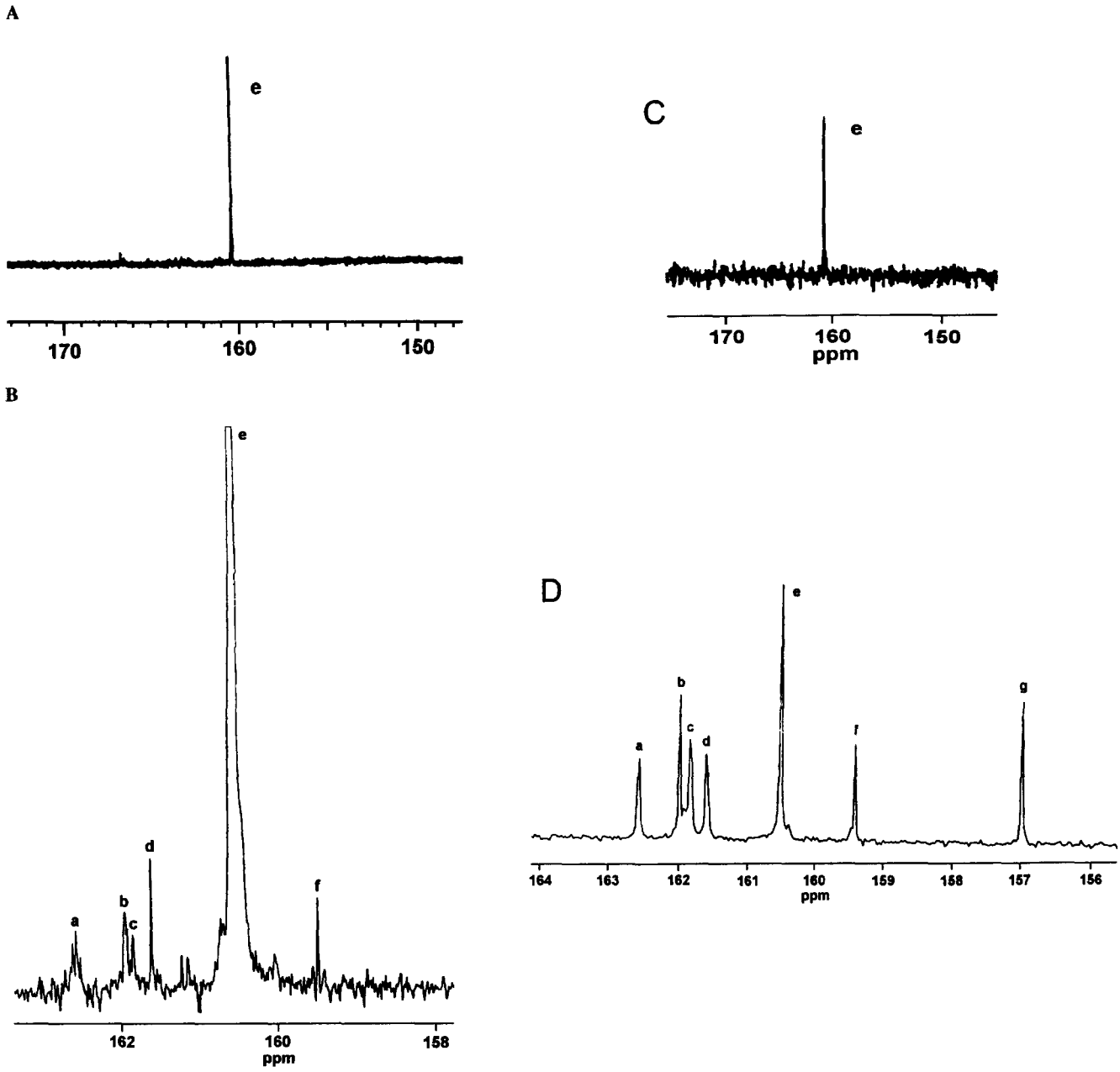

Figure 5. **A** and **B**: ^{13}C NMR of **1** isolated after feeding *rac*-[11-^{13}C]-**9** (116.5 mg in total) to five 100 mL *S. laurentii* production cultures at 24 h after inoculation. At 72 h the **1** was isolated from the cultures (11.2 mg recovered). Enrichment corresponds to 81 ± 10% ^{13}C incorporation at C-11 of the quinaldic acid moiety of **1**. **C**: ^{13}C NMR of **1** isolated after feeding [11-^{13}C]-**8** (100 mg in total) to five 100 mL *S. laurentii* production cultures at 24 h after inoculation. At 72 h the **1** was isolated from the cultures (18.0 mg recovered, although only approximately 90% pure). **D**: ^{13}C NMR of the **1** sample corresponding to **C** after 16:1 dilution with authentic **1** and subsequent repurification. Overall enrichment corresponds to 29 ± 10% ^{13}C incorporation at C-11 of the quinaldic acid moiety of **1**. Peak assignments are (see also Fig. 1): (**a**) 161.1 ppm, Thz(2) carboxyl; (**b**) 162.0 ppm, Deala(2) carboxyl; (**c**) 161.9 ppm, PipC-2; (**d**) 161.7 ppm, Thz(1) carboxyl; (**e**) 160.6 ppm, C-11 of the quinaldic acid moiety; (**f**) 159.6 ppm, Thz(4) carboxyl; and (**g**) Thz(3)C-4.[12]

exchange observed at varying concentrations of **4** and a single, saturating concentration of ATP. An apparent K_m of 3 μM was found for *rac*-**4**. Conversely, an apparent K_m value of 0.3 mM was determined for ATP at varying concentrations of ATP and a single, saturating concentration of *rac*-**4**. Apparent K_m values of 1.9 μM and 31 μM were determined for (12*S*)-**4** and (12*R*)-**4**, respectively, at saturating levels of ATP. The absolute configuration of the hydroxyethyl group of the quinaldic acid moiety of **1** is 12*S*.[9]

Of the analogues of **4** tested,[30] the hydroxymethyl derivative, **16**, was the only one that was adenylated at a measurable rate. An apparent K_m of 80 μM was measured for **16** at saturating levels of ATP and the V_{max} measured for **16** was approximately 60% of the value determined for **4**. These data show that the enzyme is highly selective for the structure and stereochemistry of substituents present at the 4-position of the quinoline system. Finally, the methyl ester, *rac*-**9**, was shown to be an effective competitive inhibitor of the adenylyl transferase activity. An apparent K_i of 28 μM was determined for *rac*-**9** at saturating levels of ATP and varying concentrations of *rac*-**4**.

Photoaffinity labeling and molecular weight estimation

The native molecular weight of the adenylyl transferase was found to be 47±4 kDa by gel permeation chromatography on a Superose 12 FPLC column, comparing its elution time to that of standard proteins of known size. An aliquot of the cell-free extract was treated with [α-^{32}P]-8-azido-ATP and exposed to UV light. The protein components of the sample were then resolved by denaturing polyacrylamide gel electrophoresis.[35] The photoaffinity-labeled proteins were detected by autoradiography (Fig. 6). A single intense band of labeled protein was found corresponding to an estimated molecular weight of 50 kDa. This band was not observed when the sample was not exposed to UV light or when an excess of unlabeled ATP was present in the incubation mixture.

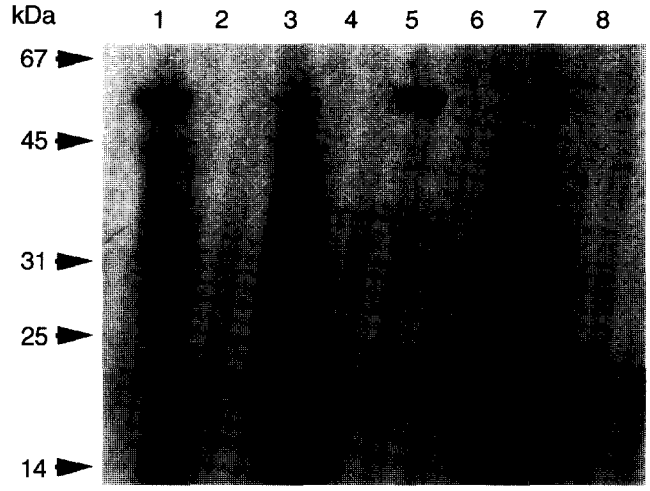

Figure 6. Autoradiogram from 12.5% SDS PAGE analysis of [α-^{32}P]-8-azido-ATP labeled cell-free extracts of *S. laurentii*. Lane 1, mixture of extract and probe exposed to UV light; lane 2, mixture of extract and probe kept in the dark; lane 3, mixture of extract, excess unlabeled ATP, and probe exposed to UV light; lane 4, mixture of extract, excess unlabeled ATP, and probe kept in the dark; lanes 5–8 are repeats of lanes 1–4 with the addition of 2-mercaptoethanol.

Discussion

The results presented above are in accord with and support the previously suggested[13,14] notion that 4-acetylquinoline-2-carboxylic acid (**3**) and 4-(1-hydroxyethyl)quinoline-2-carboxylic acid (**4**) are intermediates in the biosynthesis of the quinaldic acid moiety of **1**. The ^{13}C-label from the methyl esters [11-^{13}C]-**8** and [11-^{13}C]-**9** is specifically and efficiently incorporated at C-11 of the quinaldic acid moiety as shown by ^{13}C NMR. No significant enrichment at any other position in **1** was observed. As we had expected, the methyl esters were evidently taken up by the cells and were effectively hydrolyzed to generate the free acids inside the cell. Unexpectedly, however, the addition of the esters to the fermentation was also found to result in lower yields of the antibiotic, suggesting that **8** and **9** may inhibit a specific step or steps in the biosynthesis of **1**. Furthermore, the isotope dilution experiment demonstrated that the free acids, **3** and **4**, are present in thiostrepton-producing cultures of *S. laurentii*, although the experiment did not distinguish whether **3** and **4** were present intra- or extracellularly.

We[14] had previously proposed a mechanism for the rearrangement of the confirmed intermediate, 2-methyltryptophan,[15] or rather the corresponding keto acid, into **3** which, we suggested, is then reduced to **4**. This mechanism, shown in Figure 2, involves initial electrophilic addition to C-3 of the indole ring (Fig. 2, X^+ = electrophile). Hydrolysis of the resulting imine opens the indole ring, allowing the free amine generated from the indole nitrogen to condense with the other keto group to form the dihydroquinoline system. Elimination of X^- from the dihydroquinoline would yield the aromatic quinoline system of **3**. Formally, in this mechanism, X, which was initially introduced as X^+, is reduced to X^-. There is precedent for such a rearrangement in the work of van Tamelen and Haarsted[36] who showed that oxidation of 2-methyltryptophan with excess hypochlorite gives **3** in approximately 20% yield. The data reported above are in accord with our proposal. The alcohol **4** is present at higher concentrations in the cell, and is incorporated more efficiently into **1**, than the ketone **3**. Being a later intermediate in the proposed pathway than **3** (Fig. 2) and being closer to the final product, **4** should be more efficiently converted into **1**. Also, for pyridine nucleotide-dependent redox reactions (Fig. 2), equilibria generally favor the alcohol/NAD^+ side of the reaction rather than the ketone/NADH side. Thus, it would be expected that **4** should be present at higher concentrations in the cell than **3**.

There is, however, another plausible mechanism for the conversion of 2-methylindolepyruvate into **4**, which

cannot be ruled out based on the available data. In this mechanism (shown in Fig. 7) **3** would not be an intermediate in the formation of **4**, but rather, a shunt product. In the originally proposed pathway (Fig. 2) the intermediate dihydroquinoline eliminates X^- to form the quinoline **3**, which is converted into **4** in a separate, subsequent reduction step. Alternatively, base catalysis may lead to the loss of X^+ from the dihydroquinoline to generate an enol which could give the alcohol **3** directly. What could be the nature of X? In the original mechanism, X would be OH or a halogen, that is, the reaction would be initiated by a peroxidase or a haloperoxidase. In the alternative mechanism, X^+ may simply be a proton. Incorporation of H^+, converting the indole into the indolenine, would represent a tautomerization reaction. Loss of H^+, converting the dihydroquinoline into **4**, would be a second tautomerization reaction. An attractive feature of this mechanism is that it avoids the redox change in X, addition as X^+ and elimination as X^-, required by the previous proposal. The formation of **3**, as well as its incorporation into **1** would then be due to a (nonspecific ?) redox process interconverting **3** and **4**, which is not part of the biosynthetic pathway. An unequivocal distinction between these two mechanisms is not possible based on the presently available data, and will probably require studies at the enzymatic level.

The first event in the incorporation of the acid **4** into **1** does indeed seem to be activation of the carboxyl group as a mixed anhydride with AMP (adenylate). An enzyme activity that can catalyze this reaction has been demonstrated in, and partially purified from, cell-free extracts of the thiostrepton-producing organism, *S. laurentii*. The reaction is similar to reactions shown to occur in the biosyntheses of actinomycin,[25] triostin A,[26] quinomycin A,[26] milkamycin B,[27] and etamycin.[27] An analogous reaction was recently reported by our group, the activation of 3,4-dimethylindole-2-carboxylate to its adenylate in the biosynthesis of nosiheptide.[16] The presence of one specific protein which catalyzes the adenylation reaction in *S. laurentii* was demonstrated by photoaffinity labeling using [α-^{32}P]-8-azido-ATP. A single labeled protein band, of an estimated weight of 50 kDa, was observed by SDS PAGE analysis of protein extracts treated with the labeling reagent and UV light. The native molecular weight of the protein, estimated by gel permeation chromatography, was also found to be approximately 50 kDa, suggesting that the protein is monomeric. The adenylyl transferase is highly specific for the nature of the substituent at the 4-position of the quinoline-2-carboxylic acid and for its stereochemistry. (12*S*)-**4** but not (12*R*)-**4** is recognized by the enzyme and adenylated at a significant rate. The absolute stereochemistry of (12*S*)-**4** corresponds to the absolute configuration of the carbinol center in the quinaldic acid moiety of **1**. The slow conversion of (12*R*)-**4** observed in the kinetic analysis can be attributed to contamination of the (12*R*)-**4** substrate with (12*S*)-**4**. Subsequent experiments with a sample of (12*R*)-**4** of high enantiomeric purity showed almost no reaction (data not shown). It was also observed that *rac*-**9** was a potent competitive inhibitor of the adenylyl transfer reaction. In hindsight, that fact explains why the levels of **1** production were substantially lowered when the labeled methyl esters, **8** and **9**, were employed in the feeding experiments. Inhibition of the adenylyl transferase reduces the amount of activated **4** present in the culture and thus limits the overall amount of **1** produced. Work on the purification and further characterization of the adenylyl transferase is in progress.

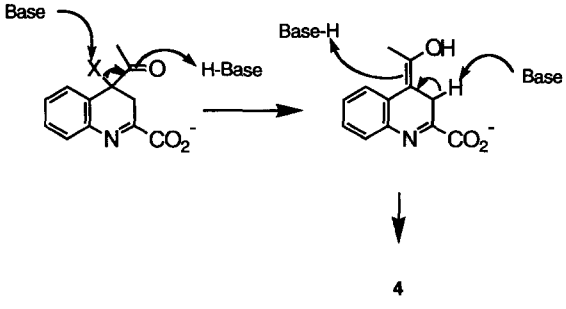

Figure 7. An alternative mechanism to account for the biosynthetic transformation of 2-methyltryptophan into **4** without a requirement for the intermediacy of **3**.

Experimental

General

Flash column chromatography ('chromatography') was done on 230–400 mesh silica gel. Elemental analyzes were carried out by the Canadian Microanalytical Service, Ltd, Delta, B.C. V4G 1G7.

The solvents $CHCl_3$ (CaH_2), CH_2Cl_2 (CaH_2), Et_2O (Na–benzophenone), MeOH ($(MeO)_2Mg$), pyridine (CaH_2) and THF (Na–benzophenone) were distilled from the respective drying agents under argon. All other solvents were HPLC grade and were used without further purification. Argon was dried by passing through a bed of 3 Å molecular sieves. Unless otherwise stated, all reagents were used as supplied by the Aldrich Chemical Co., Inc. Milwaukee.

Growth of *S. laurentii*

S. laurentii ATCC 31255 and a *S. laurentii* strain supplied by Calbiochem were stored as spore suspensions in 20% (v/v) glycerol, or as frozen samples of vegetative cultures, at −78 °C. Fermentations were carried out in a two step process. Vegetative cultures of 100 mL (15 g/L soybean flour, 50 g/L glucose, 15g/L soluble starch, pH 7.2 in tap water) were innoculated with 0.1 mL of spore suspension and grown for 48 h in 500 mL baffled Erlenmeyer flasks at 28 °C with shaking at 200 rpm (A. Kuehner Shaker Cabinet). Ten mL of vegetative culture were then used to innoculate 100 mL of production medium [50 g/L glucose, 15 g/L trypticase soy broth, 11 g/L yeast extract, 15 g/L $CaSO_4$, 1 mL/L trace elements solution (5 g/L $CoCl_2 \cdot 6H_2O$, 0.5 g/L Na_2MoO_4, 0.5 g/L H_3BO_3, 1.0 g/L $CuSO_4 \cdot 2H_2O$, 1.0 g/L $ZnSO_4 \cdot 7H_2O$), pH 7.2 in tap water] in 500 mL

baffled Erlenmeyer flasks, which were incubated at 28 °C with shaking at 200 rpm for 72–80 h.

Isolation of thiostrepton

The culture medium from ten 100 mL production cultures of *S. laurentii*, at 72 h after inoculation, was pooled and vigorously stirred with $CHCl_3$ (750 mL) to form a thick emulsion. After stirring for 30 min, the two phases were separated by centrifugation (14000 × *g*, 5 min). The aqueous phase was extracted with a further portion of $CHCl_3$ (750 mL). The combined organic extracts were dried (anhydrous Na_2SO_4), filtered, and evaporated in vacuo at below 35 °C to give a semicrystalline-yellow oil which was dissolved in a minimum volume of $CHCl_3$. Crude thiostrepton was precipitated by the addition of 10 volumes of *n*-hexane. The precipitate from this stage was dissolved in a minimal volume of CH_2Cl_2:ethanol (4:1) and reprecipitated by the addition of 5 volumes of Et_2O. Yields of thiostrepton after these precipitation steps were generally between 30 and 100 μg/mL of culture broth.

Partial purification of the adenylyl transferase

Cells from ten 100 mL production cultures of *S. laurentii* were collected by centrifugation (10000 × *g*, 4 °C) after 3–5 days of growth and suspended in 100 mL buffer A (50 mM potassium phosphate, pH 6.8, containing 5 mM dithiothreitol and 20% v/v glycerol). Phenylmethanesulfonyl fluoride (100 mM in isopropanol) was added to give a final concentration of 1 mM. The cells were disrupted by two passages through a French Press cell (Aminco, 20,000 psi) and a soluble protein fraction (90 mL) was obtained after centrifugation (25,000 × *g*, 4 °C, 30 min). Polyethyleneimine (15% w/v) was added in small portions over 10 min to the supernatant at 4 °C to give a final concentration of 0.3% w/v. The resulting precipitate was removed by centrifugation (25,000 × *g*, 4 °C, 30 min) and discarded. A slurry of DEAE-cellulose (Whatman DE52) in buffer A (23 mL) was added to the resulting solution and, after stirring for 10 min on ice, the DEAE-cellulose was removed by filtration and discarded. Powdered ammonium sulfate was added to the solution in small portions to bring the final concentration to 65% of saturation. After gentle stirring for 30 min on ice, the precipitate was collected by centrifugation (25,000 × *g*, 4 °C, 30 min) and stored at −20 °C. Prior to further work the pellet was resuspended in buffer A and the excess salt removed by gel filtration over Sephadex G-25. The enzyme retained its activity for at least six months when stored at −80 °C in buffer containing 15% (v/v) glycerol.

Adenylyl transferase activity determination

The enzyme solution (10 μL) was incubated with ATP (1 μmol), $MgCl_2$ (0.5 μmol), sodium [^{32}P]pyrophosphate (New England Nuclear, 5 nmol, ca. 500,000 cpm), **4** (0.5 μmol), and potassium phosphate (5 μmol) at pH 6.8 in a final volume of 100 μL for 10 min at room temperature. The reaction was terminated by the addition of activated charcoal (100 μL, 5% w/v suspension in water). The charcoal pellet was recovered by centrifugation and washed with water to remove excess pyrophosphate. For qualitative analysis ATP was recovered from the pellet by washing with aqueous pyridine solution (0.1 mL, 10% v/v). An aliquot of the eluate (2 μL) was analyzed by TLC on PEI-cellulose plates developed with an aqueous LiCl solution (1.5 M). The radioactivity was subsequently detected by autoradiography of the TLC plate. Quantitative analysis was performed either by directly counting another aliquot of the eluate or, more simply, by suspending the charcoal pellet in 100 μL of water and counting a 50 μL aliquot of the suspension.

Molecular weight estimation

S. laurentii cell-free extract containing adenylyl transferase activity (200 μL) was fractionated by FPLC on a Superose 12 column equilibrated, and eluted at a flow rate of 0.5 mL/min, with buffer A containing 100 mM NaCl. Fractions (0.5 mL) were collected and assayed for activity. The native molecular weight of the adenylyl transferase was estimated by comparing its elution time to that of a series of standard proteins of known molecular weight.

Photoaffinity labeling

S. laurentii cell-free extract containing adenylyl transferase activity (50 μL) was incubated with [α-^{32}P]-8-azido-ATP (ICN Biomedicals, Irvine, CA, >106 cpm), $MgCl_2$ (0.5 μmol), and **4** (0.02 μmol) in a final volume of 100 μL at room temperature. Photolabeling was initiated by exposing the samples to UV light (hand-held tlc illuminator, 254 nm, ca. 12 W, 10–15 s). In some incubations ATP (0.4 μmol) and/or 2-mercaptoethanol (0.1 μmol) were also present.

Synthesis

[11-^{13}C]-*N*-Benzoyl-2-cyano-1,2-dihydroquinoline ([11-^{13}C]-17). Freshly distilled quinoline (1.78 mL, 15 mmol) in CH_2Cl_2 (20 mL) was added to a vigorously stirred solution of K^{13}CN (0.99 g, 15 mmol, 99 + % ^{13}C) in water (8 mL). PhCOCl (2.32 mL, 20 mmol) was added dropwise and the two-phase system stirred vigorously at room temperature for 4 h. The two phases were separated and the aqueous phase was extracted with CH_2Cl_2. The combined organic extracts were washed successively with water, 5% aqueous HCl, 5% aqueous NaOH, and finally water. The organic phase was dried (anhydrous Na_2SO_4), filtered, and the solvent removed by evaporation in vacuo to give a white powder. [11-^{13}C]-**17** was obtained as white needles on recrystallization from 60% aqueous acetone (2.90 g, 75.0%): mp (from EtOAc) 151–153 °C; M+H, 262.1059; calcd for $^{12}C_{16}{}^{13}CH_{11}N_2O$, 262.1061; δ_H (300 MHz, $CDCl_2$) 6.09 (1H, ddd, $J_{1H\text{-}1H}$ = 9.1 and 6.4 Hz, $J_{1H\text{-}13C}$ = 1.2 Hz), 6.19 (1H, dd, $J_{1H\text{-}1H}$ = 6.4 Hz, $J_{1H\text{-}13C}$ = 10

Hz), 6.57 (1H, d, J=8.1 Hz), 6.83 (1H, dd, J=9.1 and 1.5 Hz), 6.92 (1H, dt, J=8.0 and 1.6 Hz), 7.09 (1H, dt, J=7.7 and 1.2 Hz), 7.22 (1H, dd, J=7.5 and 1.4 Hz), 7.27 (2H, t, J=7.1 Hz), 7.35 (2H, dd, J=7.0 and 1.3 Hz), and 7.40 (1H, tt, J=6.7 and 1.5 Hz); δ_C (75.5 MHz, $CDCl_3$) 42.15 (d, $J_{^{13}C\text{-}^{13}C}$=60 Hz), 115.71 (^{13}C-enriched, sidebands with $J_{^{13}C\text{-}^{13}C}$=60 Hz), 120.2 (d, $J_{^{13}C\text{-}^{13}C}$=3.0 Hz), 125.1, 125.6, 125.9, 127.9, 128.2, 128.3, 129.1, 129.5 (d, $J_{^{13}C\text{-}^{13}C}$=3.0 Hz), 131.4, 133.4, 134.4, and 169.1; *m/z* (FAB, $CHCl_3$-nitrobenzyl alcohol [NBA]) 262 (100%, M+H) and 234 (50%, M-^{13}CN).

[11-^{13}C]Quinoline-2-carboxylicacidhydrobromidemonohydrate ([11-^{13}C]-18). [11-^{13}C]-**17** (2.42 g, 9.3 mmol) was suspended in glacial AcOH (7.3 mL). Aqueous HBr solution (48% w/v, 7.3 mL) was added cautiously and then the yellow slurry was heated at reflux for 15 min to give a dark red–black solution. The solution was allowed to cool and then left to stand overnight. The crystals which formed were collected by filtration, washed with a little ice-cold glacial AcOH and then with Et_2O. A second crop of crystals was obtained by saturating the filtrate with Et_2O. The combined crops were dried in vacuo to give [11-^{13}C]-**18** as orange needles (2.48 g, 97.6%): M+H, 175.0588, calcd for $^{12}C_9{}^{13}CH_7NO_2$, 175.0588; δ_H (300 MHz, CD_3OD) 8.05 (1H, dt, J=7.6 and 0.94 Hz), 8.25 (1H, ddd, J=8.8, 7.0, and 1.2 Hz), 8.42 (1H, d, J=8.3 Hz), 8.53 (1H, d, J=8.6 Hz), 8.57 (1H, dd, $J_{^1H\text{-}^1H}$=8.5 Hz and $J_{^1H\text{-}^{13}C}$=2.5 Hz), and 9.33 (1H, d, J=8.5 Hz); δ_C (75.5 MHz, CD_3OD) 122.7 (d, $J_{^{13}C\text{-}^{13}C}$=4.0 Hz), 123.1, 130.4, 132.2, 132.3, 137.2, 140.5, 144.9 (d, $J_{^{13}C\text{-}^{13}C}$=76 Hz), 149.3 (d, $J_{^{13}C\text{-}^{13}C}$=3.2 Hz), and 161.8 (^{13}C-enriched, with sidebands $J_{^{13}C\text{-}^{13}C}$=76 Hz); *m/z* (FAB, thioglycol) 175 (M+H, 100%).

Methyl [11-^{13}C]quinoline-2-carboxylate ([11-^{13}C]-7). The [11-^{13}C]-**18** hydrobromide (2.35 g, 8.6 mmol) was dissolved in dry MeOH (10 mL). NaOH (360 mg, 9 mmol) was added and the resulting suspension heated at reflux until a solution was formed (ca. 15 min). The solution was cooled to room temperature and then $SOCl_2$ (3.5 mL, 47.9 mmol) was carefully added. The resulting suspension was heated at reflux for 18 h. The suspension was left to cool to room temperature and then carefully poured into aqueous, saturated $NaHCO_3$ solution. The resulting mixture was extracted repeatedly with CH_2Cl_2. The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered, and the solvent was then removed by evaporation in vacuo to give a pale-green powder that rapidly turned cream colored on exposure to air (1.06 g, 65.8%): mp (from $CHCl_3$) 81–82 °C; δ_H (300 MHz, $CDCl_3$) 4.05 (3H, d, $J_{^1H\text{-}^{13}C}$=3.8 Hz), 7.63 (1H, dt, J=7.6 and 0.7 Hz), 7.75 (1H, ddd, J=8.6, 7.0, and 1.4 Hz), 7.84 (1H, dd, J=7.7 and 1.1 Hz), 8.16 (1H, d, J=8.5 and 1.5 Hz), 8.27 (2H, d, J=8.5 Hz); δ_C (75.5 MHz, $CDCl_3$) 53.13 (d, $J_{^{13}C\text{-}^{13}C}$ = 2 Hz), 121.0 (d, $J_{^{13}C\text{-}^{13}C}$ = 7.4 Hz), 127.5, 128.6, 129.3, 130.2, 130.6, 137.2 (d, $J_{^{13}C\text{-}^{13}C}$ = 4.2 Hz), 147.5, 147.8 (d, $J_{^{13}C\text{-}^{13}C}$ = 84 Hz), and 165.9 (^{13}C-enriched, with sidebands of $J_{^{13}C\text{-}^{13}C}$ = 84 Hz).

Methyl [11-^{13}C]-4-acetylquinoline-2-carboxylate ([11-^{13}C]-8). CAUTION: *This reaction can be quite exothermic. Acetaldehyde boils at room temperature and is toxic. Care should be taken to sufficiently cool the reaction flask and the condenser. The reaction should be carried out in a well-ventilated fume hood.* $FeSO_4 \cdot 7H_2O$ (1.57 g, 5.7 mmol) in water (10 mL) and aqueous H_2O_2 solution (10 mL; 5.7 mmol) were added separately and simultaneously, over a 5 min period, to a stirred solution of [11-^{13}C]-**7** (1.06 g, 5.7 mmol) and TFA (0.44 mL, 5.7 mmol) in acetaldehyde (40.6 mL, 0.73 mol). The addition of the reagents caused the solvent to reflux vigorously. After 5 min the pale-orange solution was diluted with water (40 mL) and extracted with CH_2Cl_2. The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered, and then the solvent removed by evaporation in vacuo to give a yellow-brown powder. [11-^{13}C]-**8** was obtained by chromatography on silica gel, eluting with EtOAc:hexanes (1:1), as a pale-yellow powder (1.06 g, 81.3%): mp 119–121 °C (from EtOAc–hexanes); δ_H (200 MHz, $CDCl_3$) 2.66 (3H, s), 3.96 (3H, d, $J_{^1H\text{-}^{13}C}$= 3.8 Hz), 7.56 (1H, ddd, J=8.4, 6.9, and 1.5 Hz), 7.67 (1H, ddd, J=7.7, 7.55, and 1.6 Hz), 8.17 (1H, dd, J=8.4 and 1.0 Hz), 8.26 (1H, d, $J_{^1H\text{-}^{13}C}$=1.7 Hz), 8.35 (1H, dd, J=8.2 and 1.3 Hz); δ_C (75.5 MHz, $CDCl_3$) 29.70, 53.07, 119.7 (d, $J_{^{13}C\text{-}^{13}C}$=8.0 Hz), 124.5, 125.2, 130.1, 130.3, 130.8, 143.6 (d, $J_{^{13}C\text{-}^{13}C}$=83.8 Hz), 148.3, 165.1 (^{13}C-enriched, with sidebands of $J_{^{13}C\text{-}^{13}C}$=83.8 Hz), 200.2.

Methyl *rac*-[11-^{13}C]-4-(1-hydroxyethyl)quinoline-2-carboxylate (*rac*-[11-^{13}C]-9). $NaBH_4$ (84.7 mg, 2.2 mmol) was added, as a powder, to a stirred solution of [11-^{13}C]-**8** (0.51 g, 2.2 mmol) in dry MeOH (40 mL) at room temperature. After 20 min the excess borohydride was destroyed by the addition of saturated aqueous NH_4Cl solution (ca. 50 mL). The resulting solution was extracted with CH_2Cl_2. The organic extracts were pooled, dried (anhydrous Na_2SO_4), filtered, and the solvent then removed by evaporation in vacuo to give a pale-yellow oil. The racemic alcohol was then obtained by chromatography on silica gel, eluting with EtOAc:hexanes (2:1), as a white powder (0.38 g, 74.6%): δ_H (200 MHz, $CDCl_3$) 1.51 (3H, d, J=6.5 Hz), 3.87 (3H, d, $J_{^1H\text{-}^{13}C}$=3.7 Hz), 4.06 (1H, br s), 5.54 (1H, q, J = 6.5 Hz), 7.45 (1H, ddd, J=8.3, 6.9, and 1.3 Hz), 7.58 (1H, ddd, J=8.4, 6.9, and 1.3 Hz), 7.87 (1H, dd, J=8.4 and 0.8 Hz), 8.09 (1H, s), 8.11 (1H, d, J=8.6 Hz); δ_C (75.5 MHz, $CDCl_3$) 24.28, 52.82 (d, $J_{^{13}C\text{-}^{13}C}$=2.1 Hz), 63.91, 116.5 (d, $J_{^{13}C\text{-}^{13}C}$=7.4 Hz), 122.8, 128.2, 129.6, 130.9, 147.2 (d, $J_{^{13}C\text{-}^{13}C}$=8.2 Hz), 147.3 (d, $J_{^{13}C\text{-}^{13}C}$=84 Hz), 153.4 (d, $J_{^{13}C\text{-}^{13}C}$ =3.2 Hz), and 165.6 (^{13}C-enriched, sidebands with J=80 Hz).

4-Acetylquinoline-2-carboxylic acid (3). A 30% (w/v) aqueous NaOH solution (1.33 mL, 10 mmol) was added to a stirred solution of **8** (250 mg, 1.1 mmol) in THF (3.67 mL) at room temperature. The solution immediately turned yellow and a thick-yellow precipitate formed within 5 min. After 100 min the precipitate was dissolved by the addition of 0.1 N NaOH solution (50 mL). The solution was washed twice with CH_2Cl_2

and then acidified to pH 1 with dilute aqueous HCl. The product was extracted with EtOAc. The organic extracts were pooled, dried over anhydrous Na_2SO_4, filtered, and the solvent removed by evaporation in vacuo to give a pale-green crystalline solid. The acid was then further purified by recrystallization from EtOAc–hexanes (128.3 mg, 54.7%): mp 290 °C dec; ν_{max} (KBr) 3400 (N—H str), 1693 (C=O str), 1610, and 1458 cm^{-1} (deprot acid C=O str); λ_{max} (H_2O) 248 (6000 $M^{-1}cm^{-1}$) and 322 nm (2304); δ_H (300 MHz, $CDCl_3$) 2.80 (3H, s), 7.78 (1H, dt, $J=7.7$ and 1.3 Hz), 7.88 (1H, dt, $J=7.6$ and 1.4 Hz), 8.21 (1H, d, $J=8.8$ Hz), 8.49 (1H, s), 8.56 (1H, d, $J=8.4$ Hz).

[11-^{13}C]-4-Acetylquinoline-2-carboxylic acid ([11-^{13}C]-3). This compound was prepared as required from [11-^{13}C]-**8** by hydrolysis employing the procedure used for the unlabeled material.

***rac*-4-(1-Hydroxyethyl)quinoline-2-carboxylic acid (*rac*-4).** *rac*-**9** (562 mg, 2.0 mmol) was dissolved in THF (8 mL). Aqueous 30% (w/v) NaOH solution (1.7 mL) was added and the two-phase system stirred at room temperature. After 2 h the white precipitate was collected by filtration, washed with THF, and recrystallized from EtOH to give the sodium salt of the acid as a white powder (345 mg, 76.6%): mp 182 °C (evacuated); ν_{max} (Nujol) 1610 and 1400 cm^{-1} (deprot acid C=O str); λ_{max} (MeOH) 242 (6430 $M^{-1}cm^{-1}$), 286 (2960), 310 (2200), and 322 nm (1750); δ_H (300 MHz, $CDCl_3$) 1.68 (3H, d, $J=6.6$ Hz), 5.70 (1H, q, $J=6.5$ Hz), 7.71 (1H, ddd, $J=7.8$, 7.6, and 1.3 Hz), 7.82 (1H, ddd, $J=8.4$, 7.0, and 1.3 Hz), 8.14 (1H, d, $J=8.7$ Hz), 8.18 (1H, d, $J=8.0$ Hz), 8.48 (1H, s); δ_C (75.5 MHz, DMSO-d_6) 24.7, 66.8, 117.8, 124.4, 127.1, 128.1, 130.3, 131.2, 148.6, 154.4, 157.0, and 173.4.

***rac*-[11-^{13}C]-4-(1-Hydroxyethyl)quinoline-2-carboxylic acid (*rac*-[11-^{13}C]-4).** This compound was prepared as required from *rac*-[11-^{13}C]-**9** by hydrolysis employing the procedure used for the unlabeled material.

Methyl *rac*-4-[1-(butanoyloxy)ethyl]quinoline-2-carboxylate (*rac*-10). Dry pyridine (1.46 mL, 18 mmol) and *n*-PrCOCl (1.71 mL, 16.5 mmol) were added to a stirred solution of *rac*-**9** (2.31 g, 10 mmol) in dry CH_2Cl_2 (25 mL). The solution was stirred at room temperature for 3 h. A white precipitate formed. The suspension was diluted with CH_2Cl_2 (25 mL) and then washed, successively, twice with saturated aqueous $CuSO_4$ solution, once with water and once with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to give a brown oil. The butyrate ester was obtained by chromatography on silica gel, eluting with EtOAc:hexanes (1:1), as a white crystalline solid (2.97 g, 98.7%): mp (from EtOAc–hexanes) 68–69 °C; Found: C, 67.7; H, 6.3; N, 4.7%. $C_{17}H_{19}NO_4$ requires: C, 67.8; H, 6.3; N, 4.65%; M+H, 302.1395; requires 302.1392; ν_{max} (CH_2Cl_2 solution) 2962 (C—H str), 1734 (C=O str), 1731 cm^{-1} (C=O str); λ_{max} (MeOH) 246 (9066 $M^{-1}cm^{-1}$) and 294 nm (5420); δ_H (300 MHz, $CDCl_3$) 0.82 (3H, t, $J=7.4$ Hz), 1.57 (3H, d, $J=6.6$ Hz), 1.57 (2H, sext, $J=7.4$ Hz), 2.29 (2H, t, $J=7.4$ Hz), 3.95 (3H, s), 6.50 (1H, q, $J=6.6$ Hz), 7.54 (1H, ddd, $J=8.4$, 6.9, and 1.2 Hz), 7.65 (1H, ddd, $J=8.4$, 7.0, and 1.3 Hz), 7.96 (1H, d, $J=8.3$ Hz), 8.15 (1H, s), 8.21 (1H, d, $J=8.1$ Hz); δ_C (75.5 MHz, $CDCl_3$) 13.31, 18.21, 21.45, 35.99, 52.88, 67.52, 116.9, 122.5, 125.9, 128.6, 129.7, 131.4, 147.6, 148.8, 165.6, 172.3.

Methyl *rac*-4-[1-(acetoxy)ethyl]quinoline-2-carboxylate (12). Prepared as for **10** from acetyl chloride (0.35 mL, 5 mmol) and *rac*-**9** (0.46 g, 2.0 mmol). Yield 461.1 mg, 84.5%: mp (from CH_2Cl_2) 103–104 °C; ν_{max} (CH_2Cl_2 solution) 1740 (C=O) and 1737 cm^{-1} (C=O); λ_{max} (MeOH, nm) 220 (4190 M^{-1} cm^{-1}), 248 (6200), 294 nm (4140); δ_H (300 MHz, $CDCl_3$) 1.66 (3H, d, $J=6.6$ Hz), 2.14 (3H, s), 4.05 (3H, s), 6.58 (1H, q, $J=6.6$ Hz), 7.65 (1H, ddd, $J=8.2$, 7.1, and 1.2 Hz), 7.75 (1H, dt, $J=7.7$ and 1.3 Hz), 8.05 (1H, d, $J=8.4$ Hz), 8.23 (1H, s), 8.30 (1H, d, $J=8.3$ Hz); δ_C (75.5 MHz, $CDCl_3$) 21.13, 21.71, 53.20, 67.99, 117.1, 122.7, 126.1, 128.8, 130.0, 131.7, 147.8, 147.8, 148.8, 166.0, 170.0; *m/z* (Direct Probe EI-MS) 273 (19%, M^+), 231 (28), 215 (100), 154 (60), 128 (42).

Methyl *rac*-4-[1-(caproyloxy)ethyl]quinoline-2-carboxylate (*rac*-13). Prepared as for **10** from freshly distilled caproyl chloride (0.39 mL, 2.5 mmol) and *rac*-**9** (234 mg, 1.0 mmol). Colorless oil (328 mg, 90.7%): δ_H (300 MHz, $CDCl_3$) 0.55 (3H, t, $J=6.7$ Hz), 0.96 (8H, m), 1.34 (2H, m), 1.40 (3H, d, $J=6.7$ Hz), 2.14 (2H, t, $J=7.4$ Hz), 3.78 (3H, s), 6.34 (1H, q, $J=6.6$ Hz), 7.34 (1H, ddd, $J=8.5$, 7.0, and 1.3 Hz), 7.46 (1H, ddd, $J=8.3$, 7.1, and 1.2 Hz), 7.80 (1H, d, $J=8.2$ Hz), 8.00 (1H, s), 8.04 (1H, d, $J=8.3$ Hz); δ_C (75.5 MHz, $CDCl_3$) 13.33, 21.00, 21.87, 24.28, 28.22, 28.36, 30.93, 33.70, 52.33, 67.16, 116.6, 122.2, 125.5, 128.2, 129.3, 131.0, 147.2, 147.3, 148.4, 165.1, 171.9.

Methyl (*R*)-4-(1-hydroxyethyl)quinoline-2-carboxylate [(12*R*)-9]. *Candida cylindracea* lipase (Sigma, EC 3.1.1.3, 0.7 mg, 12.1 kU) was added to a stirred emulsion of *rac*-**10** (67.3 mg, 0.22 mmol) in 100 mM potassium phosphate buffer, pH 7.0 (1.0 mL) and DMSO (0.1 mL). The resulting emulsion was stirred at 50 °C for 3 days with frequent shaking being required to disperse any lumps that formed. The incubation mixture was then diluted with brine and extracted with EtOAc. The organic phase was dried over anhydrous Na_2SO_4, filtered, and the solvent removed by evaporation in vacuo to give a colorless oil. The starting material **10** (48.9 mg, 72.5%) and (12*R*)-**9** (4.3 mg, 8.2%) were obtained by chromatography on silica gel, eluting with EtOAc:hexanes (1:1). (12*R*)-**9** was identical to the previously prepared *rac*-**9**. The enantiomeric purity of the product was determined by ^{1}H NMR analysis of its (1*S*)-camphanate derivative (de of 92.6±0.5%).

Methyl 4-[1-(benzoyloxy)ethyl]quinoline-2-carboxylate (11). DEAD (34.5 μL, 0.22 mmol) was added dropwise over 2 min to a solution of **9** (25.2 mg, 0.11 mmol), PhCOOH (30.6 mg, 0.25 mmol), and Ph_3P

(60.1 mg, 0.23 mmol) in dry CH_2Cl_2 (1.0 mL). The resulting colorless solution was stirred at room temperature for 1.5 h before being diluted with CH_2Cl_2 (2 mL) and washed with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to give a colorless oil. The product was obtained by chromatography on silica gel, eluting with EtOAc:hexanes (1:1), as a white crystalline compound (36.2 mg, 99.1%): mp 114–118 °C; ν_{max} (CH_2Cl_2) 1720 cm^{-1} (C=O str); M+H, 336.1219; $C_{20}H_{17}NO_4$+H requires 336.1236; λ_{max} (MeOH) 244 (18600 $M^{-1}cm^{-1}$) and 294 nm (4500); δ_H (200 MHz, $CDCl_3$) 1.83 (1H, d, J=6.6 Hz), 4.06 (3H, s), 6.85 (1H, q, J=6.5 Hz), 7.46 (1H, dt, J=6.8 and 1.5 Hz), 7.57 (2H, m), 7.70 (1H, dt, J=6.9 and 1.4 Hz), 7.80 (1H, dt, J=6.9 and 1.5 Hz), 8.11 (2H, dd, J=7.8 and 1.6 Hz), 8.18 (1H, d, J=8.2 Hz), 8.34 (1H, d, J=8.4 Hz), 8.36 (1H, s); δ_C (75.5 MHz, $CDCl_3$) 21.8, 53.2, 68.7, 117.3, 122.8, 126.3, 128.1, 128.5, 129.1, 129.68, 129.73, 130.2, 131.6, 133.4, 147.7, 149.1, 165.6, 165.8.

(2*S*)-Mosher ester of methyl 4-(1-hydroxyethyl)quinoline-2-carboxylate (14). (2*R*)-MTPA chloride (237 mg, 1.0 mmol) in dry CCl_4 (1.0 mL) was added to a stirred solution of *rac*-**9** (154 mg, 0.66 mmol) and dry pyridine (80 μL, 1.0 mmol) in dry CCl_4 (5 mL). The resulting solution was stirred for 22 h at room temperature and then diluted with CH_2Cl_2 (25 mL). The solution was washed twice with saturated aqueous $CuSO_4$ solution, once with water, and finally once with saturated aqueous $NaHCO_3$ solution. The organic phase was dried (anhydrous Na_2SO_4), filtered, and evaporated in vacuo. The oil obtained was purified by chromatography on silica gel, eluting with EtOAc:hexanes (4:1), to afford the (2*S*)-Mosher ester of **9** as a colorless oil (281.7 mg, 94.5%): M+H, 448.1358, $C_{23}H_{20}NO_5F_3$+H requires, 448.1372; δ_C (75.5 MHz, $CDCl_3$; a mixture of diastereoisomers) 13.93, 20.72, 21.11, 21.32, 52.86, 52.95, 55.25, 55.27, 55.39, 55.41, 60.09, 70.51, 70.53, 85 (br m), 117.3, 117.6, 122.4, 122.5, 125.6, 125.7, 126.9, 127.1, 128.2, 128.3, 128.8, 128.9, 129.4, 129.5, 129.9, 130.1, 131.5, 131.6, 131.6, 146.9, 147.0, 147.7, 147.8, 165.5, 165.6; δ_F (282.39 MHz, $CDCl_3$; a mixture of diastereoisomers) 78.1 and 78.3; *m/z* (FAB, $CHCl_3$-NBA) 448 (M+H, 100%), 329 (10), 230 (10), 215 (30), 189 (55).

(2*S*)-Mosher ester of (12*R*)-9. δ_H (300 MHz, $CDCl_3$) 1.73 (3H, d, J=6.6 Hz), 3.54 (3H, d, J=1.0 Hz), 3.96 (3H, s), 6.78 (1H, m), 7.25 (3H, m), 7.35 (2H, m), 7.61 (1H, m), 7.76 (1H, m), 8.02 (1H, d, J=8.2 Hz), 8.06 (1H, s), 8.34 (1H, d, J=8.3 Hz).

(2*S*)-Mosher ester of (12*S*)-9. δ_H (300 MHz, $CDCl_3$) 1.66 (3H, d, J=6.5 Hz), 3.43 (3H, d, J=1.0 Hz), 3.99 (3H, s), 6.81 (1H, m), 7.25 (3H, m), 7.35 (2H, m), 7.66 (1H, m), 7.79 (1H, m), 8.07 (1H, d, J=8.2 Hz), 8.25 (1H, s), 8.37 (1H, d, J=8.3 Hz).

(1*S*)-Camphanate ester of methyl 4-(1-hydroxyethyl)-quinoline-2-carboxylate. (1*S*)-(−)-Camphanic acid chloride (500 mg, 2.31 mmol) and dry pyridine (0.185 mL, 2.29 mmol) were added to a stirred solution of *rac*-**9** (213.3 mg, 0.92 mmol) in dry $CHCl_3$ (3.0 mL). The solution was stirred at room temperature for 20 h and then diluted with $CHCl_3$. The organic phase was washed successively, twice with saturated aqueous $CuSO_4$ solution, twice with 1 N HCl, and twice with saturated aqueous $NaHCO_3$ solution. The organic phase was then dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to give a pale-yellow oil. TLC analysis showed that conversion to the ester had occurred quantitatively. Purification by chromatography on silica gel, eluting with EtOAc:hexanes (1:1), gave a pale-yellow oil (336.1 mg, 88.6%): mp (from EtOAc:hexanes) 112–113.5 °C; Found C, 67.0; H, 6.05; N, 3.45%, $C_{23}H_{25}NO_6$ requires C, 67.1; H, 6.1; N, 3.4%; M+H, 412.1755, requires 412.1760; ν_{max} (neat) 3059 (C—H str), 2971 (C—H str), 1790, 1746, 1724 cm^{-1} (C=O str); λ_{max} (MeOH) 246 (10 900 $M^{-1}cm^{-1}$) and 296 nm (4790); *m/z* (FAB, $CHCl_3$-NBA) 412 (15%, M+H), 329 (5), 307 (20), 289 (10), 214 (7), 176 (12).

(1*S*)-Camphanate ester of (12*R*)-9. δ_H (300 MHz, $CDCl_3$) 0.90 (3H, s), 1.03 (3H, s), 1.10 (3H, s), 1.70 (1H, m), 1.77 (3H d, J=6.7 Hz), 1.90 (1H, m), 2.10 (1H, m), 2.45 (1H, m), 4.06 (3H, s), 6.72 (1H, q, J=6.6 Hz), 7.70 (1H, ddd, J 8.4, 7.4, and 1.1 Hz), 7.80 (1H, dt, J=7.2 and 1.1 Hz), 8.10 (1H, d, J=8.4 Hz), 8.25 (1H, s), 8.37 (1H, d, J=8.4 Hz); δ_C (75.5 MHz, $CDCl_3$) 9.65, 16.76, 16.85, 21.73, 28.96, 29.69, 30.90, 53.27, 54.54, 54.90, 69.77, 90.83, 117.40, 122.71, 126.1, 129.14, 130.28, 131.85, 147.92, 147.96, 165.70, 166.88, 177.90.

(1*S*)-Camphanate ester of (12*S*)-9. δ_H (300 MHz, $CDCl_3$) 0.98 (3H, s), 1.03 (3H, s), 1.10 (3H, s), 1.70 (1H, m), 1.78 (3H, d, J=6.7 Hz), 1.90 (1H, m), 2.10 (1H, m), 2.45 (1H, m), 4.07 (3H, s), 6.71 (1H, q, J=6.6 Hz), 7.70 (1H, ddd, J = 8.4, 7.4, and 1.1 Hz), 7.80 (1H, dt, J=7.2 and 1.1 Hz), 8.10 (1H, d, J=8.4 Hz), 8.27 (1H, s), 8.37 (1H, d, J=8.4 Hz); δ_C (75.5 MHz, $CDCl_3$) 9.65, 16.71, 16.85, 21.62, 28.93, 29.69, 30.80, 53.27, 54.54, 54.90, 69.77, 90.83, 117.40, 122.71, 126.1, 129.14, 130.28, 131.85, 147.92, 147.96, 165.70, 166.88, 177.90.

(12*R*)- and (12*S*)-4-(1-Hydroxyethyl)quinoline-2-carboxylic acid [(12*R*)- and (12*S*)-4]. Diastereomerically pure (1*S*)-camphanate ester of (12*R*)- or (12*S*)-**9** (4.8 mg, 11.8 mmol, >98% de) was dissolved in THF (0.33 mL). Aqueous 30% NaOH solution (0.17 mL) was added and the two-phase system stirred at 28 °C for 2.5 h. The reaction mixture was neutralized and the precipitate dissolved in dilute HCl. The solution was made up to exactly 5.0 mL and then stored frozen prior to use in the enzymatic studies.

Methyl 4-hydroxymethylquinoline-2-carboxylate (15). TFA (1.8 mL, 24 mmol) was added carefully to a solution of $FeSO_4 \cdot 7H_2O$ (0.67 g, 2.4 mmol) and **7** (4.5 g, 24 mmol) in MeOH (150 mL) and the solution brought to reflux. Aqueous H_2O_2 (11.1 mL, 48.4 mmol)

was carefully added over a period of 30 min to give a pale-brown solution which was heated at reflux for a further 15–18 h. The solution was left to cool to room temperature and then concentrated in vacuo. The residue (ca. 50 mL) was diluted with water (250 mL) and extracted with $CHCl_3$. The organic extracts were pooled, dried (anhydrous Na_2SO_4), filtered, and the solvent evaporated in vacuo to give a dark-brown oil. The product **15** was obtained by chromatography on silica gel, eluting with EtOAc:hexanes (4:1), as a pale-yellow oil (1.97 g, 52.8% based on recovered starting material {1.29 g}): mp (from EtOAc–hexanes) 128–129.5 °C; M+H, 218.0821, $C_{12}H_{11}NO_3$+H requires, 218.0817; ν_{max} (CH_2Cl_2 solution) 3 168 (O—H str), 2993 (C—H str), and 1731 cm^{-1} (C=O str); λ_{max} (MeOH) 244 (12,050 $M^{-1}cm^{-1}$) and 292 nm (4 680); δ_H (300 MHz, $CDCl_3$) 4.07 (3H, s), 5.25 (2H, s), 7.65 (1H, ddd, J= 8.4, 6.9, and 1.3 Hz), 7.77 (1H, ddd, J=8.6, 6.8, and 1.4 Hz), 8.0 (1H, dd, J=8.5 and 1.0 Hz), 8.29 (1H, s), and 8.32 (1H, d, J=8.1 Hz); δ_C (75.5 MHz, DMSO-d_6) 52.54, 59.47, 117.09, 123.44, 126.24, 128.53, 130.12, 130.38, 146.58, 147.48, 149.88, 165.51; *m/z* (FAB, $CHCl_3$-NBA) 218 (M+H, 100%), 176 (66), and 165 (25).

4-Hydroxymethylquinoline-2-carboxylic acid (16). Aqueous NaOH solution (30%, 1.33 mL, 10 mmol) was added to a stirred solution of **15** (0.57 g, 2.63 mmol) in THF (3.67 mL) at room temperature. A yellow precipitate formed immediately. After 10 min **16** was recovered by filtration and washed with THF (0.52 g, 87.9%): mp 240 °C dec; ν_{max} (KBr) 3421 (N—H str), 1617, and 1458 cm^{-1} (deprot acid C=O str); λ_{max} (H_2O) 242 (5670 $M^{-1}cm^{-1}$) and 292 nm (1620); δ_H (200 MHz, D_2O) 5.00 (2H, s), 7.49 (1H, ddd, J=8.4, 6.9, and 1.2 Hz), 7.65 (1H, ddd, J=8.5, 6.9, and 1.4 Hz), 7.79 (1H, s), 7.81 (1H, d, J=7.0 Hz), and 7.92 (1H, d, J=8.2 Hz); δ_C (75.5 MHz, D_2O) 61.7, 118.8, 124.3, 127.1, 129.0, 129.5, 130.1, 131.4, 147.3, 149.5, and 155.6.

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29. In the present assay only apparent K_m and V_{max} values could be measured, as 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 (ATP and **4**) are present in excess.

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31. The following compounds were tested as substrates and found not to be adenylated at detectable rates using the present assay procedure: **3**, **15**, **16**, 3,4-dimethylindole-2-carboxylate,[32] and quinoxaline-2-carboxylate.[33]

32. 3,4-Dimethylindole-2-carboxylate plays a similar role to **4** in the biosynthesis of nosiheptide in *Streptomyces actuosus*.[16,34]

33. Quinoxaline-2-carboxylate plays a similar role to **4** in the biosynthesis of triostin A and quinomycin A in *Streptomyces triostinicus*.[26]

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