An Abstract of The Thesis of

W. Randal Erickson for the degree of Doctor of Philosophy in Chemistry presented July 29, 1987

Title: Studies On Advanced Intermediates In The Biosynthesis of Streptonigrin

Redacted for privacy

Abstract Approved: Dr. Steven J. Gould

Early studies on the biosynthesis of the quinoline quinone portion of streptonigrin, **1**, had suggested the intermediacy of novel aromatic amino acids. In order to test this hypothesis, a series of compounds was synthesized and tested for potential incorporation. [4-^{15}N] 4-Aminoanthranilic acid, **45a**, [4-^{2}H] 7-aminoquinaldinic acid, **51a**, and [4-^{2}H] 7-amino-5-hydroxyquinaldinic acid, **53a**, were synthesized and fed to fermentations of *Streptomyces flocculus*. Incorporation of **45a** and **51a** but not **53a** provided suggestive evidence that 4-aminoanthranilic acid is a new product of the shikimate pathway and provided an example of a fundamentally new route for the biological formation of the quinoline ring system.

Several possibilities also existed with regard to the source and timing of the oxygenation of the A and D rings of **1**. A fermentation in the presence of $^{18}O_2$ gas revealed that molecular oxygen was the source of all the A and D ring oxygen atoms and, along with the above results, suggested that these oxidations occur at a late stage in the biosynthesis.

STUDIES ON ADVANCED INTERMEDIATES IN THE BIOSYNTHESIS OF STREPTONIGRIN

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A Thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for
the degree of

Doctor of Philosophy

Completed July 29, 1987

Commencement June 1988

Approved:

Redacted for privacy

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Date Thesis Is Presented July 29, 1987

To Kathy

Acknowledgments

I would like to thank my research director Dr. Steven J. Gould for his constant support and occassional prodding, without which I could not have completed this work. Thanks also go to all the other members of the chemistry community at Oregon State University who have helped me throughout my time here.

Special thanks go to Rodger Kohnert for providing the mass spectral and NMR services as well as allowing me to terrorize his vineyards. Thanks also to my friend and colleague John Wityak for providing additional NMR services and advice.

I would also like to thank my good friend and one-time roommate Todd Somers for all of his expert advice, solicited or otherwise, as well as all the good times. Thanks also to all the members of my research group with special acknowledgments to Dr. V. A. Palaniswamy and Dr. Larry R. Hillis.

Outside the realm of chemistry I would like to thank, first and foremost, my mother for her love, advice, wisdom, and financial support. Tremendous thanks also go out to my very good friends Bill Komparda and Keith Leo and their families for their constant love over the years.

In closing I would like to thank my future bride, Kathy, for all the love and understanding she has shown as well as putting up with my bickering and complaining during my graduate career.

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STUDIES ON ADVANCED INTERMEDIATES IN THE BIOSYNTHESIS OF STREPTONIGRIN

Introduction

Streptonigrin (STN), **1**, (Fig. 1) was first isolated by Rao and Cullen in 1959 as a metabolite of *Streptomyces flocculus* (ATCC 13852)[1]. It is also produced by a number of other *Streptomyces* species including *S. albus* var. *bruneomycini* (hence named bruneomycin)[2], *S. echinatus* and *S. rufochromogenes* 662 (hence named rufochromycin)[3]. More recently it was found to be produced by *S. retrostaticus* as a co-metabolite with retrostatin[4].

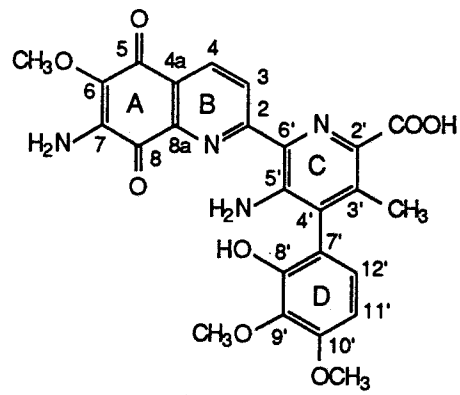

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Figure 1. Streptonigrin

The unique tetracyclic pyridylquinoline quinone structure of **1** has been rigorously established through spectral and degradative methods[5] as well as X-ray crystallography[6]. The latter revealed that the A, B, and C rings are nearly coplanar while the D ring is roughly orthogonal. Streptonigrin is optically active and is suggested to have the S configuration[7].

Other streptonigrin-like metabolites have been discovered in the past few years (Fig. 2). Umezawa and co-workers initially reported the isolation of 6-*O*-demethylstreptonigrin, **2**[8], but later revised its structure to 10'-*O*-demethylstreptonigrin, **3**[9], from a strain of *S. albus* MG 883-12F2. Lavendamycin, **4**[10], produced by *S. lavendulae* and streptonigrone, **5**[11], produced by an unidentified species of *Streptomyces* also belong to this class.

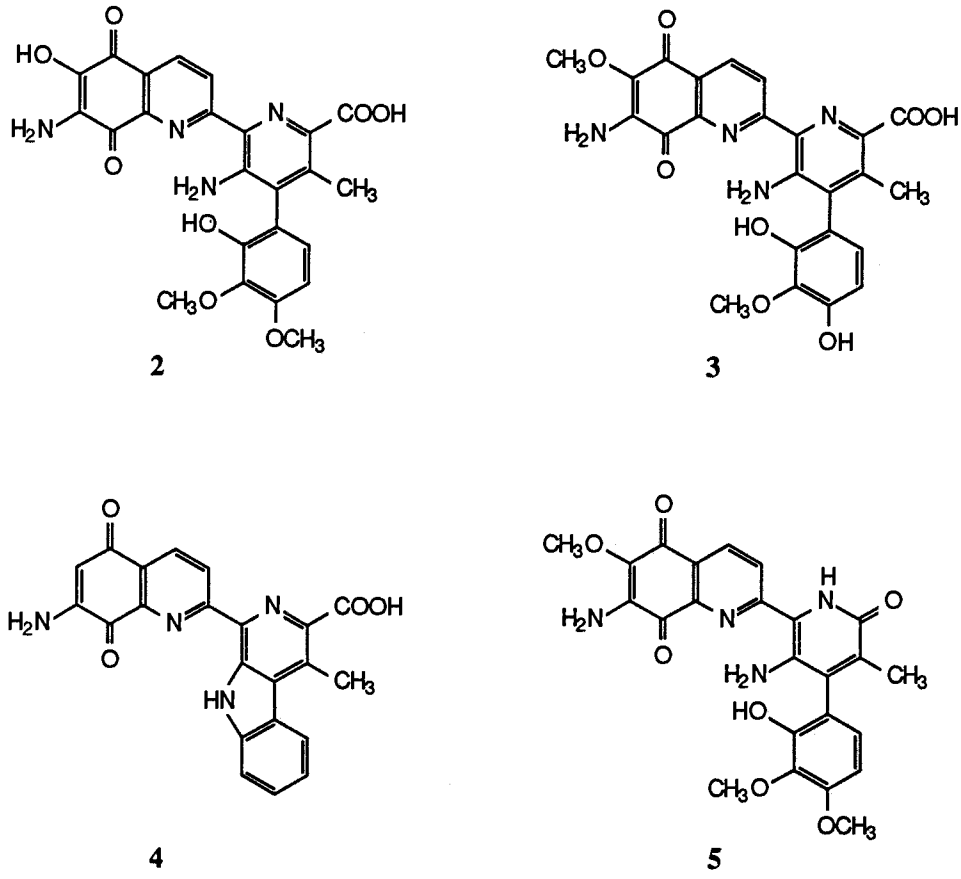


Figure 2. Streptonigrin-like Metabolites

Streptonigrin was shown to have antibiotic and anticancer activity by researchers at Chas. Pfizer, Inc. and the Sloan-Kettering Institute. Its clinical effectiveness is limited, however, due to its toxicity in moderate doses[12,13].

The naturally occurring 10'-*O*-demethylstreptonigrin is approximately twenty times weaker in cytotoxicity against P388 leukemia cells *in vitro* than **1**, having an ID_{50} of 0.58 μg / mL. Lavendamycin had no inhibitory effect on P388 but did show antimicrobial activity against a variety of organisms[14]. In virtually every case **4** was less potent than **1**, typically giving values two to ten times lower.

Many streptonigrin analogs have been synthesized[15] and tested for activity. Chemical modifications of the amino group at C-7 or C-5' or the hydroxyl group at C-8' of **1** usually resulted in substantial loss of antitumor

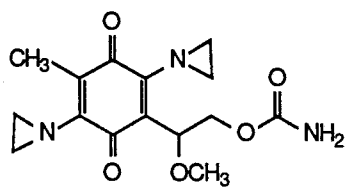
activity[16]. Elaboration at the carboxylate moiety with various amines[17] gave amides (Table 1) which all showed a marked decrease in antibacterial activity[18,19]. Some of these derivatives showed rather strong cytotoxicity against mouse lymphomas. The methyl ester of STN, **6**, showed some chemotherapeutic improvement[20,21] and had one fourth the cytotoxicity of streptonigrin. This could have been due to partial hydrolysis to **1**, however. Streptonigrin hydroxamic acid, **12**, and streptonigrin hydrazide, **13**, showed weak antimicrobial activities as determined by the agar dilution method. The remaining derivatives had lost virtually all activity against both Gram-positive and Gram-negative bacteria. All of the derivatives did show varying cytocidal activities against murine lymphosarcoma L5178Y cells. Compounds **6**, **7**, **10**, and **13** were also shown to be active as electron acceptors in the oxidation of NADH by *Clostridium kluyveri* diaphorase[22]. Streptonigrin was also recognized to have inhibitory activities of reverse transcriptase although this was lost in its methyl ester **6**[23]. The remaining derivatives all retained this activity[18].

6	STN-OCH_3
7	STN-NH_2
8	STN-$NHCH_2CH_2OH$
9	STN-$NHCH_2CH_2CH_2N(CH_3)_2$
10	STN-$NHCH_2COOH$
11	STN-$NHCH_2COOCH_3$
12	STN-NHOH
13	STN-$NHNH_2$
14	STN-$NHNHCONH_2$
15	STN-$NHNHCSNH_2$
16	STN-$NH(CH_2)_3NH(CH_2)_4NH$-STN

Table 1. Structures of Streptonigrin Derivatives

Mechanism of Action

During the early investigations on the mode of action of streptonigrin it was postulated[24,25] that in low concentrations of **1** a catalytic oxidation of NADPH by a mitochondrial diaphorase occurred followed by subsequent generation of hydrogen peroxide and a concomitant decrease in ATP synthesis as oxidative phosphorylation was bypassed. Single strand breaks in DNA via radicals were also considered because at higher concentrations **1** exhibited an initial first order decline in viability implying that a single hit per cell was lethal and the bacterial chromosome was the most probable site of action. It was also shown that streptonigrin's greatest lethal effect occured in the presence of an electron source, oxygen, and certain divalent metal ions[26]. The interaction of metal ions with STN and the biological properties of these complexes have been extensively studied[27,28].



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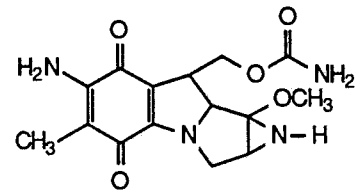

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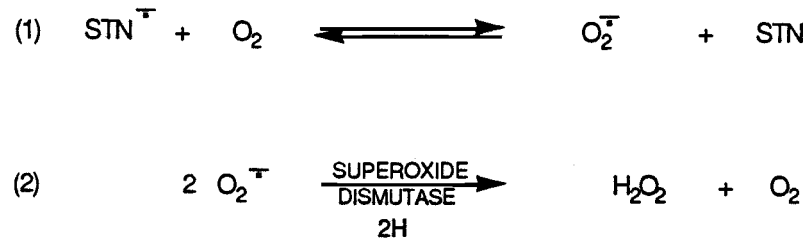
Figure 3. Other Hydroxyl Radical Generating Aminoquinones

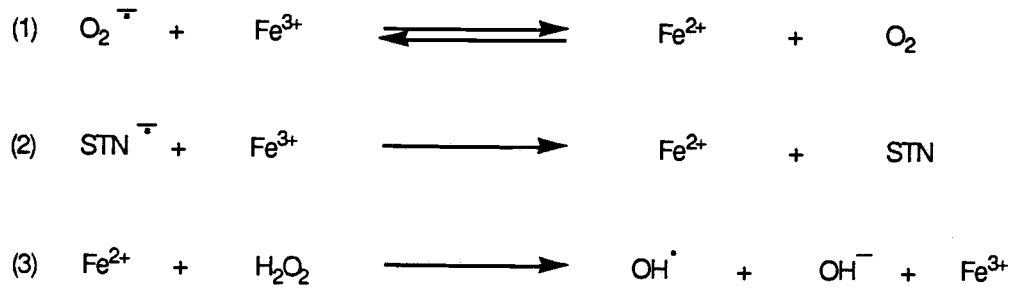
In the presence of oxygen the reduced form of **1**, as well as those of the aminoquinones carboquone, **17** and mitomycin C, **18**, (Fig. 3) generate hydroxyl radicals[29,30]. These hydroxyl radical generations were inhibited by superoxide dismutase and catalase, suggesting that the hydroxyl radicals originate from superoxide anion radicals ($O_2^{\cdot}$). The disproportionation of O_2

forms hydrogen peroxide which in the presence of trace amounts of iron leads to the formation of hydroxyl radicals[31].

STN induced deoxyribose degradation in cell-free systems is dependent on both the reduction of the drug to a semiquinone and on traces of molecular oxygen[32]. This degradation was also strongly inhibited by superoxide dismutase, catalase, and a variety of hydroxyl radical scavengers. Iron salts also appear to play some part in radical formation as it was partially inhibited by desferrioxamine. Thus under normal oxygen concentration the following mechanism was proposed:



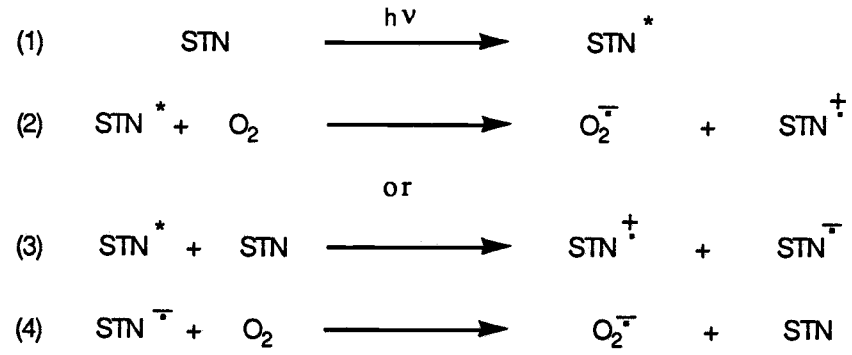
Under depleted oxygen concentration the sequence shown below was proposed:



There is also evidence that STN exerts its interference with cell respiration and cell replication in the presence of oxygen and cuprous ions[33,34]. In DNA breakage by the STN·Cu(II)·NADPH system some preference toward cleavage at cytosine bases adjacent to purine bases such

as GCGG (5'-3'), ACGG (5'-3'), and GGCG (5'-3') sequences was seen[35]. Proton chemical shifts of a STN·Cu(I)·poly (dA-dT) complex demonstrated an interaction between the C ring of **1** and purine bases of the nucleic acid.

Recent work[36] has shown that visible light (405-615 nm) excitation of STN, carboquone, and mitomycin C dissolved in dimethylsulfoxide in the presence of oxygen generated superoxide ion radicals as detected by ESR via spin trapping with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). The photogeneration of the superoxide radical most likely occurs via a direct electron transfer from the photoexcited drug molecules to dissolved oxygen. Possible reaction sequences similar or identical to ones previously described were proposed:



The same workers showed that ultraviolet radiation (313 ± 10 nm) of these aminoquinones in the presence of peptides resulted in the decarboxylation of the peptides specifically at the C-terminal amino acid. The photoexcited drugs are reduced, abstracting an electron from the carboxyl group of the peptide.

Structure/Activity Relationships

After initial studies on STN's mechanism of action the minimal requisite partial structure, **20**, (Fig. 4) was proposed[16,37]. It was recently shown[38] that the 5,8-quinoline-quinone is the minimum entity for inhibition of reverse transcriptase activity. Two compounds, 6-methoxy-5,8-dihydroquinoline-5,8-dione, **21**, and 6-methoxy-7-methyl-5,8-

dihydroquinoline-5,8-dione, **22**, (Fig. 4) were synthesized[39] and tested for both cytocidal and reverse transcriptase inhibition activity. The results indicated that while these compounds showed full inhibition of reverse transcriptase activity, they did not fully display the cytotoxicity of streptronigrin.

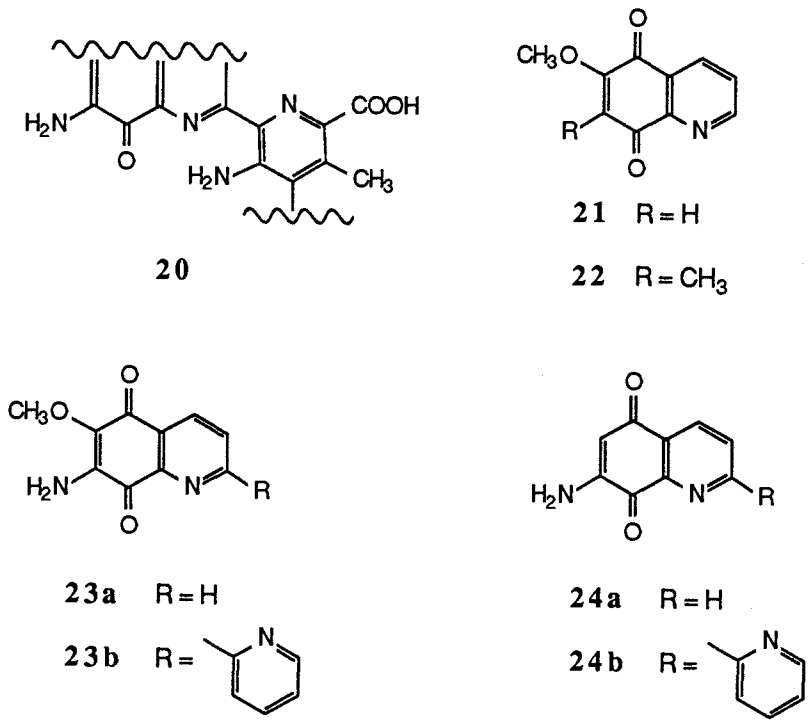


Figure 4. Minimal Requisite Structure and Quinoline Quinone Analogs

Other workers synthesized and tested[40] the quinoline quinone compounds **23a**, **23b**, **24a**, and **24b** (Fig. 4) which are identical to the AB ring systems of streptonigrin and lavendamycin. *In vitro* cytotoxic activity against L1210 and 9PS gave ID_{50} values of 0.34 and 0.19 μg/mL, respectively, for **23a**, 0.42 and 0.0023 μg/mL, respectively, for **23b**, 1.0 and 0.4 μg/mL, respectively, for **24a**, and not tested and 0.034 μg/mL, respectively, for **24b**. ID_{50} values for streptonigrin were 0.61 and 0.0045 μg/mL, respectively. Studies on related pyridylquinoline quinones also showed activity[41].

Other recent work[42] showed similar results. A series of aza- and diazanaphthoquinones (22 compounds) related to the AB ring system were synthesized and tested *in vitro* for their ability to degrade DNA. A quantitative linear relationship between the reduction potentials and the rate of DNA degradation was observed. Almost all the synthetic compounds were superior to STN in DNA degrading ability.

The C-2' carboxyl group is also a necessary entity as evidenced by the complete lack of antimicrobial activity[11] of streptonigrone **5** in disc assays at 50 μg/mL against strains of *S. aureofaciens*, *S. fragilis*, *Bacillus subtilus*, *Escherichia coli* and *Sacchromyces cerivisiae* on seeded agar plates. STN inhibits all these test organisms. This is in perfect agreement with earlier findings[43] implicating the carboxylic acid moiety as a requirement.

Biosynthetic Methodology

In the study of the biosynthesis of secondary metabolites several criteria must be met for a compound to be established as an intermediate[44]. First, it must be shown that the compound in question is incorporated into the final product and that this occurs in a specific fashion. Second, it must be proven that the compound is produced by the organism during a typical fermentation. These criteria are typically tested by performing 1) a feeding experiment and 2) an isotope trapping experiment as described *vide infra.*

Feeding Experiments

A typical feeding experiment entails sterile administration of the suspected intermediate labelled with an isotope suitable for detection in the final metabolite. In the majority of cases today, the isotope is either deuterium (^{2}H) or carbon-13 (^{13}C) and is detected by nuclear magnetic resonance spectroscopy (NMR). However, other stable isotopes such as ^{18}O and ^{15}N are also frequently employed; the former, being transparent to NMR (spin = 0), is detected through its effect on an adjacent ^{13}C resonance frequency. Mass spectroscopy is also frequently used to detect incorporation but is less reliable in determining the exact location of the label. When practical and synthetically feasible a small amount of radioactive precursor

(usually ^{3}H or ^{14}C labelled) is co-administered to aid in determination of incorporation values.

It should be noted that a labelled precursor may be incorporated into the final product but not be a legitimate precursor. The compound fed may not have been distinguishable from the normal substrate by the enzymes, and therefore a novel biosynthesis induced. A reasonable biosynthesis requires examination of all available evidence.

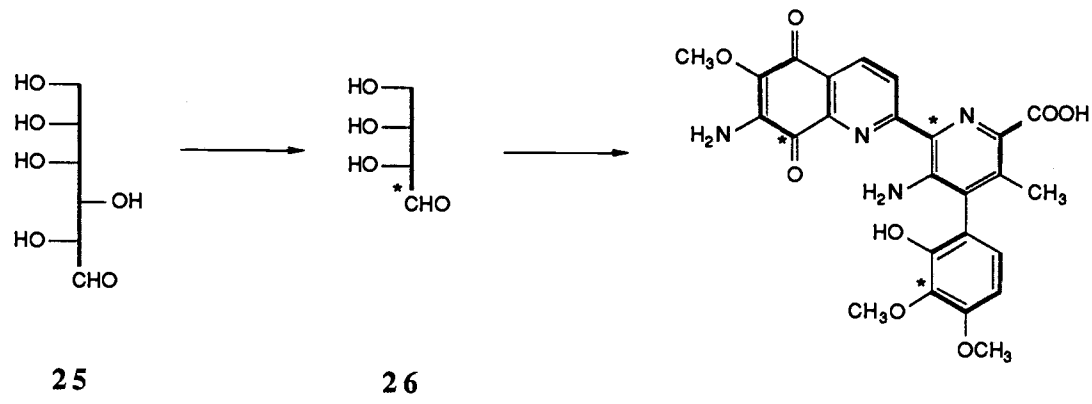
Isotope Trapping Experiments

In a typical experiment a primary precursor which is known to be incorporated into the final metabolite is used. This precursor is radioactive (^{3}H or ^{14}C) and must have a high specific activity to insure that after dilution and incorporation values are taken into account enough radioactivity remains to be detected. The labelled precursor is fed and the fermentation terminated shortly thereafter. A significant quantity (50-100 mg) of unlabelled synthetic carrier (the putative intermediate) is then added to the fermentation. In theory this material will admix with any biosynthesized labelled material. The compound (or derivative thereof) is then isolated from the fermentation broth and recrystallized to a constant specific molar radioactivity. To be considered statistically valid, the minimum radioactivity detected in a given sample must be twice that of background.

Previous Biosynthetic Studies Of Streptonigrin

The primary precursors of streptonigrin have been well established in these laboratories. Initial studies on the periphery of **1** revealed that the three *O*-methyl groups and the *C*-methyl group are derived either directly from methionine or indirectly from serine.[45]. It was also calculated that the *C*-methylation occurs much earlier than *O*-methylation in STN biosynthesis.

Studies utilizing uniformly labelled [^{13}C] glucose, **25**[46], and [1-^{13}C] erythrose, **26**[47], revealed these sugars to be excellent precursors, giving the labelling pattern shown (Fig. 5). The heavy lines indicate where intact carbon units derived from glucose are specifically incorporated into streptonigrin.



25 26

Figure 5. Labelling Pattern of STN from Glucose and Erythrose

This labelling pattern is very suggestive of a shikimate/anthranilate type biogenesis of streptonigrin. Therefore shikimic acid itself was tested as a precursor[48]. No label was observed in the derived STN from [U-^{14}C]shikimic acid. However the bulk of the radioactivity administered remained in the fermentation broth indicating poor uptake. Anthranilic acid labelled at COOH was also fed but again no incorporation was seen. At the time it was difficult to interpret these findings.

Eventually the biosynthesis of the phenylpicolinic acid framework (C and D rings) was deduced[49] as being derived from L-tryptophan, **27**, via β-methyltryptophan, **28**, as shown in Figure 6. The correct stereochemistry of **28** was proven to be 2S,3R by two different research groups[49b,50]. It was also ingeniously shown[49a,c] that the C-5' amine nitrogen was derived from the indole nitrogen of tryptophan by the use of doubly labelled [1-^{15}N, 2-^{13}C] tryptophan. This result, which represents the first biological cleavage of its kind, was detected by the observation of a doublet flanking the ^{13}C resonance of C-5' in the ^{13}C NMR spectrum and was confirmed by the presence of a doublet in the ^{15}N NMR spectrum. Interestingly tryptophan was not incorporated into the quinoline quinone portion as initially expected.

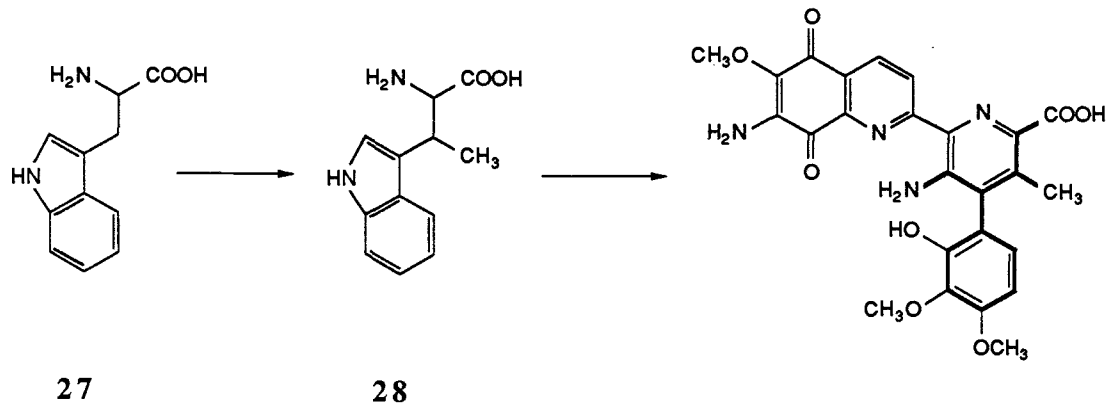


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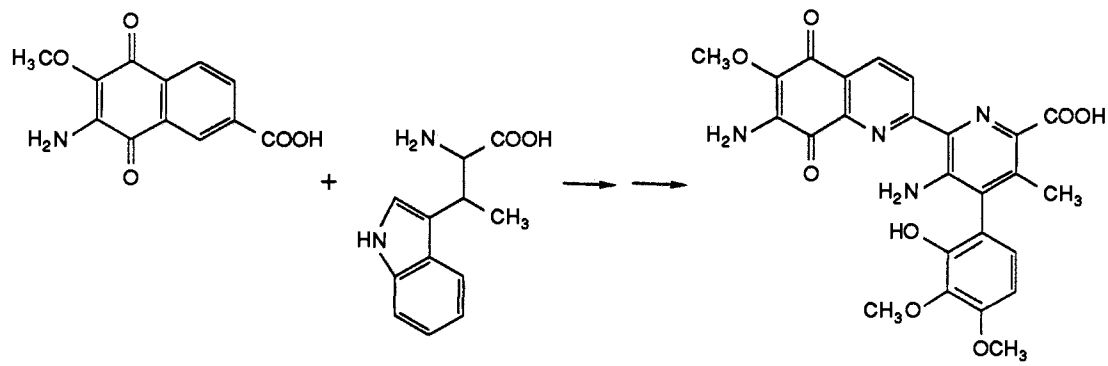
Figure 6. Incorporation of Tryptophan and β-Methyltryptophan into STN

Rationale of Present Studies

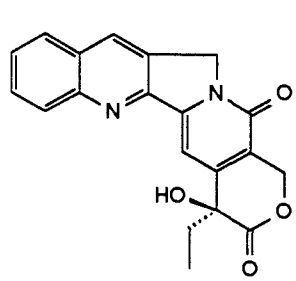
As previously mentioned, the biosynthesis of the C and D rings of streptonigrin had been rigorously established. The missing portion could formally be thought of as the equivalent of the substituted quinoline-2-carboxylic acid **29** which could condense with β-methyltryptophan, **28**, and subsequently cyclize and be oxidized to STN (Scheme 1). The hypothesis that the D-ring oxidations occur after this condensation was supported by the late timing of *O*-methylation and the isolation of lavendamycin, **4**, which contains an intact β-carboline unit unsubstituted in the benzene ring. The timing of oxidations of the A ring at the C-5 and C-8 positions were likewise unkown but the oxidation at the C-6 position most likely occurred after the condensation, again for the same reasons previously mentioned.



29 **28** **1**

Scheme 1. Coupling of Biogenic Equivalents

Initially it was thought that the quinoline quinone system might be derived via metabolism of tryptophan as is well documented for a host of other natural quinoline containing compounds including camptothecin, **30**[51], xanthommatin, **31**[52], kynurenic acid, **32**, xanthurenic acid, **33**, quinaldic acid, **34**, and 8-hydroxyquinaldic acid, **35**[53] (Fig. 7). This degradation proceeds through kynurenine or 3-hydroxykynurenine as depicted in Scheme 2. However, the complete lack of incorporation of tryptophan ruled out this pathway.



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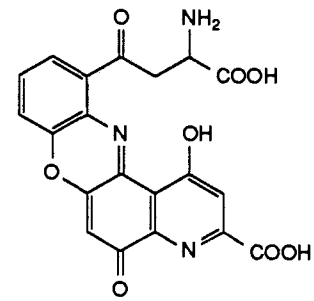

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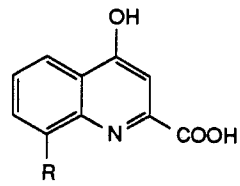
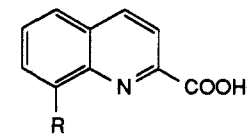


32 R = H
33 R = OH



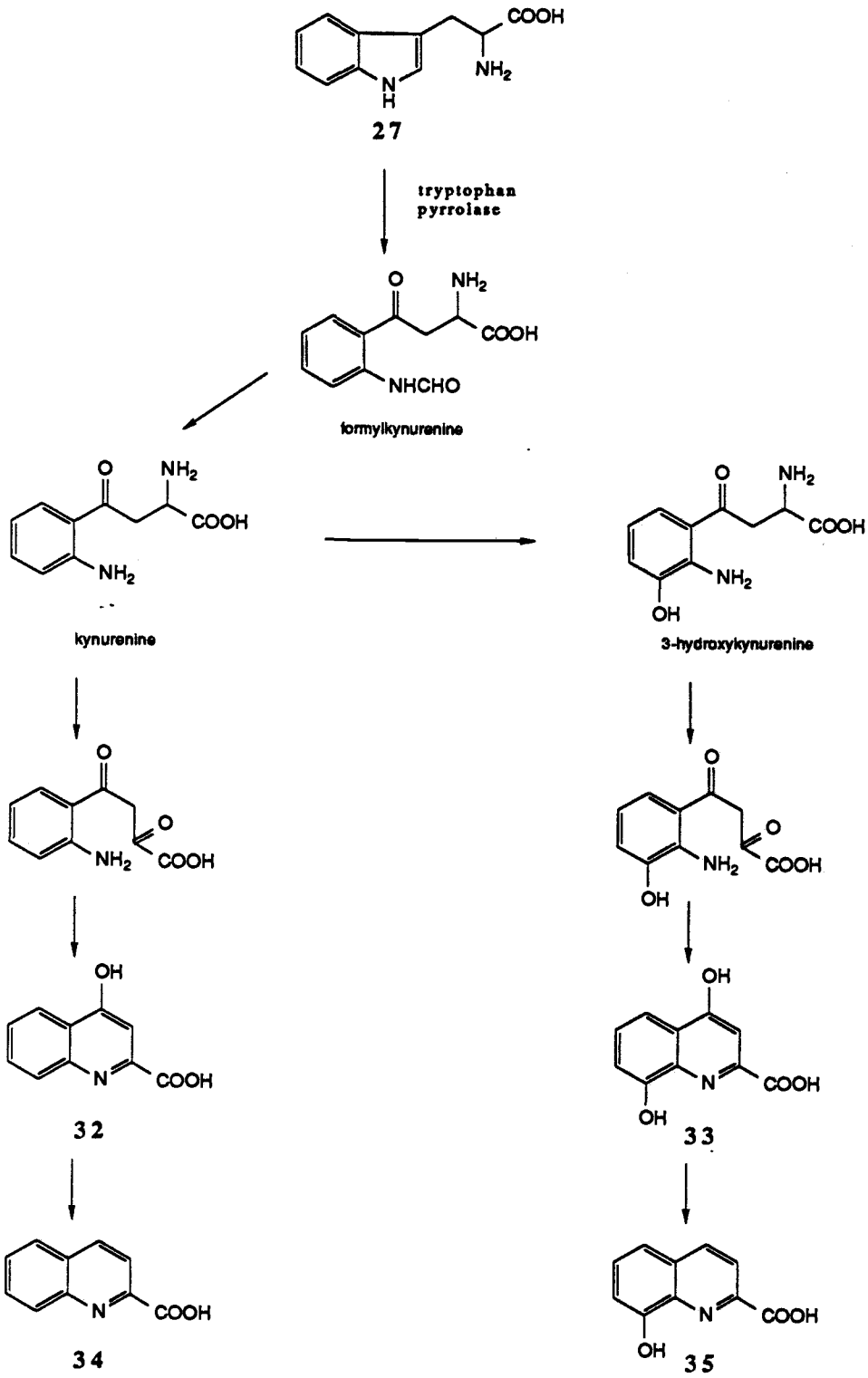
34 R = H
35 R = OH

Figure 7. Quinoline Natural Products Derived from Tryptophan

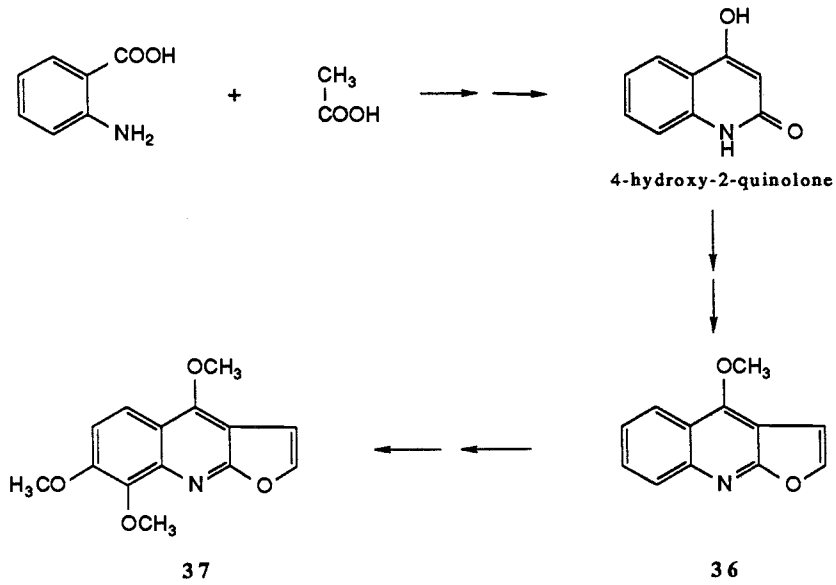
Quinoline ring systems are also biosynthesized[54] via anthranilic acid and "acetate" as exemplified by dictamnine, **36**, and skimmianine, **37** (Scheme 3). This pathway was also ruled out as in these cases the carboxyl group of anthranilic acid is retained.

The [4+2] labelling pattern of the A ring from [U-^{13}C] glucose (Fig. 5) suggested that a shikimate-type metabolite was an intermediate. We therefore hypothesized that decarboxylation of an anthranilate may occur during the biosynthesis of the quinoline ring. It is chemically feasible that condensation of a substituted aromatic amino acid with erythrose or its equivalent with concomitant decarboxylation would afford the required quinoline-2-carboxylate skeleton.

Natural products which are derived from aromatic amino acids (Fig. 8) include pactamycin, **38**[55], from 3-aminobenzoic acid, **39**, actinocin, **40**[56], and cinnabarinic acid, **41**[57], from 3-hydroxyanthranilic acid, **42**, and



Scheme 2. Tryptophan Metabolism Pathway



Scheme 3. Quinolines via Anthranilate and "Acetate"

rifamycin, **43**[55], and mitomycin, **17**[55], (see Fig. 3) from 3-amino-5-hydroxybenzoic acid, **44**.

On biogenic grounds we felt that both amine groups should be introduced at the pre-aromatic stage. We felt that two amino acids, 2,4-diaminobenzoic acid, **45**, (also refered to as *m*-aminoanthranilic acid) and 2,4-diamino-3-hydroxybenzoic acid, **46**, at the time not known to be natural products, were two logical choices (Fig. 9). Recently, 6-hydroxyanthranilic acid, **47**, was determined to be a natural product and a precursor to sarubicin, **48**[58] (Fig. 8). By analogy 2,4-diamino-6-hydroxybenzoic acid, **49**, (Fig. 9) potentially could have been a precursor to STN. It will be shown, however, that it was highly unlikely that **49** was involved.

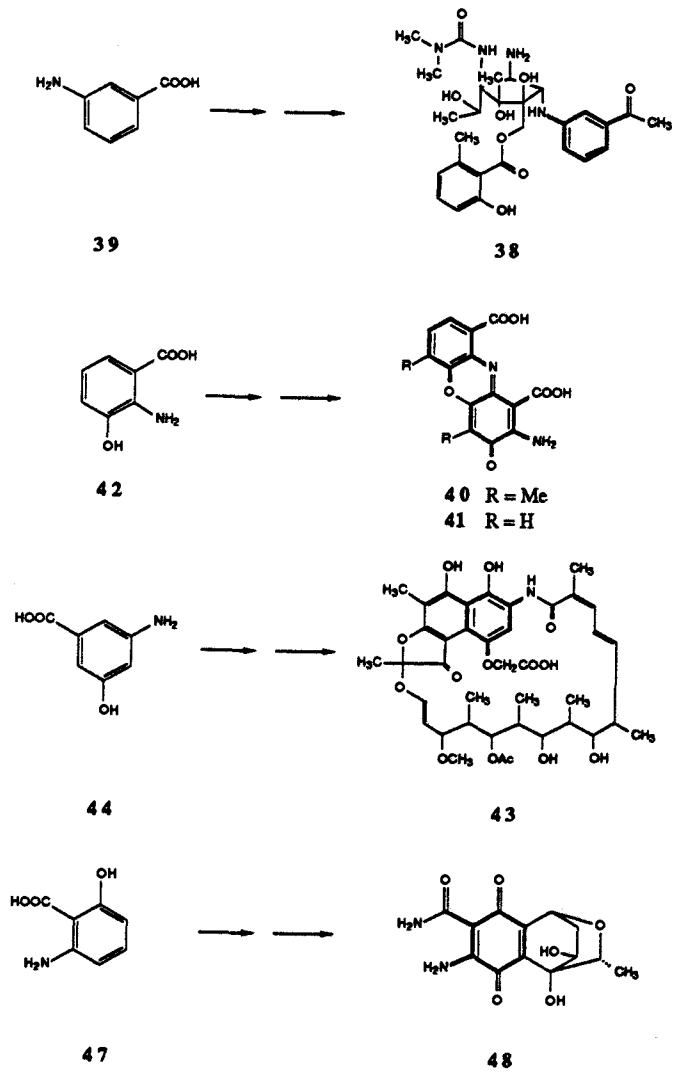


Figure 8. Natural Products Derived From Aromatic Amino Acids

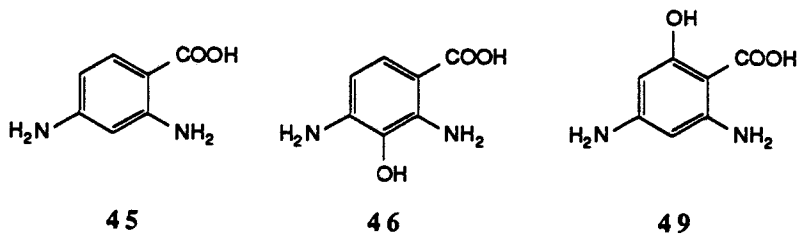
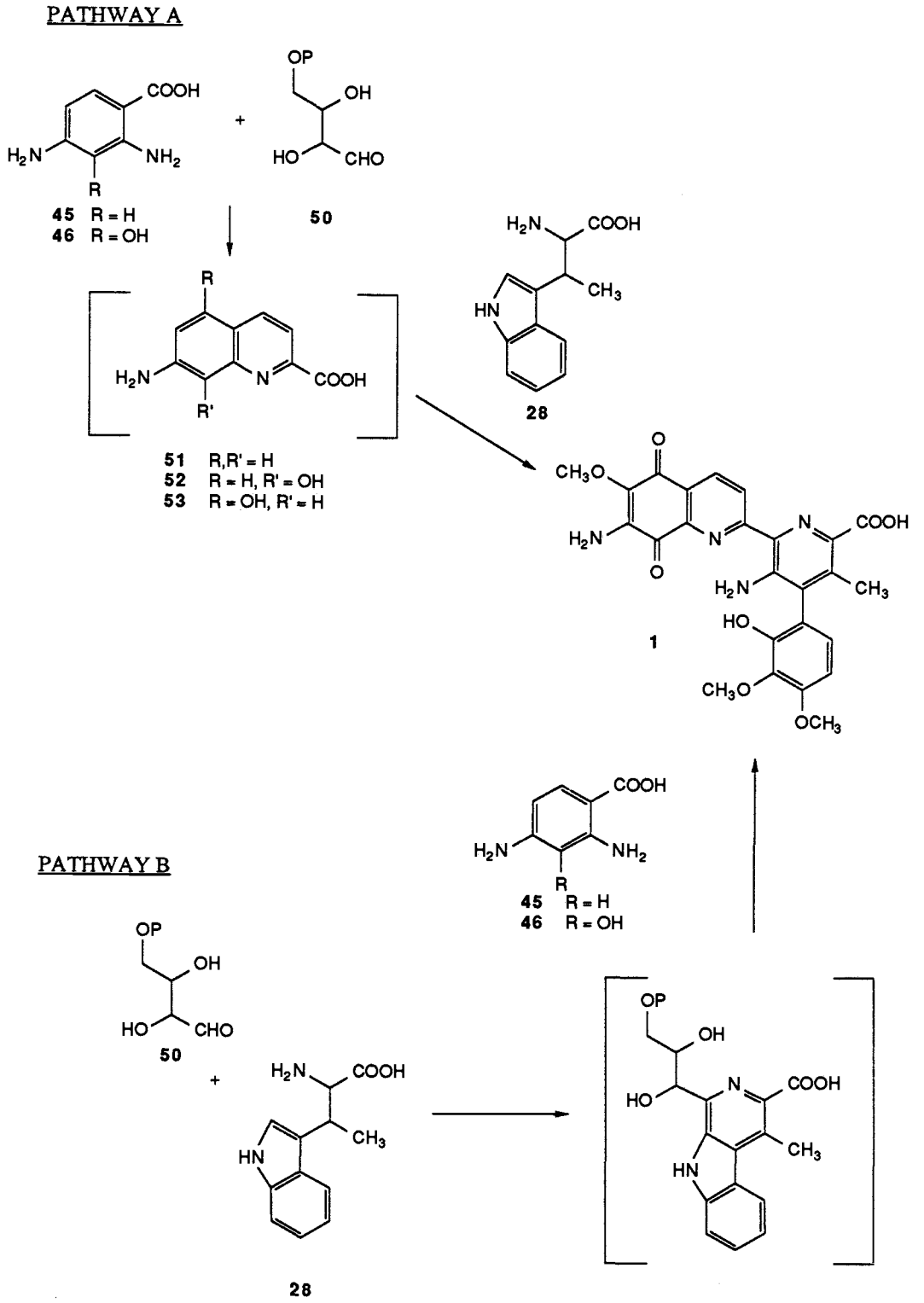


Figure 9. Potential Anthranilate Precursors of the A Ring of STN

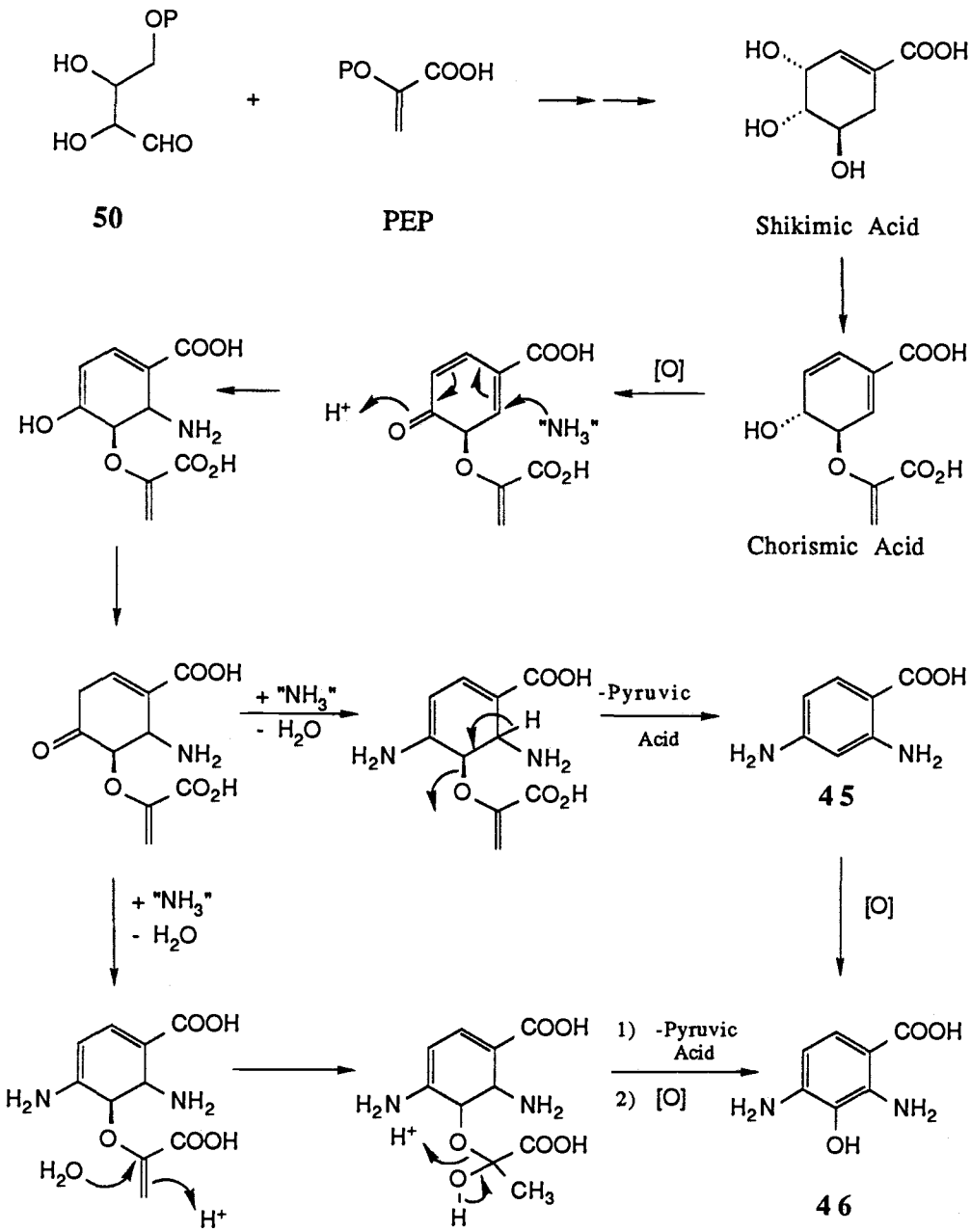
Assuming one or both of these amino acids, **45** and **46**, to be involved there were two possible biosynthetic pathways leading to streptonigrin (Scheme 4). Pathway A entailed condensation of an anthranilate with erythrose-4-phosphate, **50**, to form an aminoquinaldinic acid, **51** or **52**, which could couple with **28**. Alternatively in Pathway B the erythrose-4-phosphate could first couple with β-methyltryptophan forming an intermediate β-carboline. This could then cyclize with the aminoanthranilic acid. The main thrust of my research focused on the investigation of Pathway A. Therefore the main compounds of interest initially were **45**, **46**, 7-aminoquinaldinic acid, **51**, and 7-amino-8-hydroxyquinaldinic acid, **52**. 7-Amino-5-hydroxyquinaldinic acid, **53**, was later added to this list of potential intermediates.

Plausible biosyntheses of the aminoanthranilates **45** and **46** are depicted in Scheme 5. The nitrogen source is represented as "NH_3" for convenience. The biogenesis of **45** is straightfoward; however, **46** may be derived directly from **45** by a simple oxidation, as is the case for 3-hydroxyanthranilic acid, **42**[59],in some organisms, or through vinyl ether hydrolysis of a chorismate-like intermediate, as is proposed for isochorismate in the biosynthesis of 2,3-dihydroxybenzoic acid[60]. The important difference between these two pathways shown is the source of the hydoxyl oxygen. If **45** is the precursor to **46**, the hydroxyl group would be derived from molecular oxygen. However if only **46** were involved then this hydroxyl group must necessarily have been retained from the original pre-aromatic precursor, erthyrose-4-phosphate. A third possibility of the anthranilate **45** being a viable precursor but not **46**, requires that this oxygen be introduced at a much later stage. The source would necessarily be molecular oxygen or water.

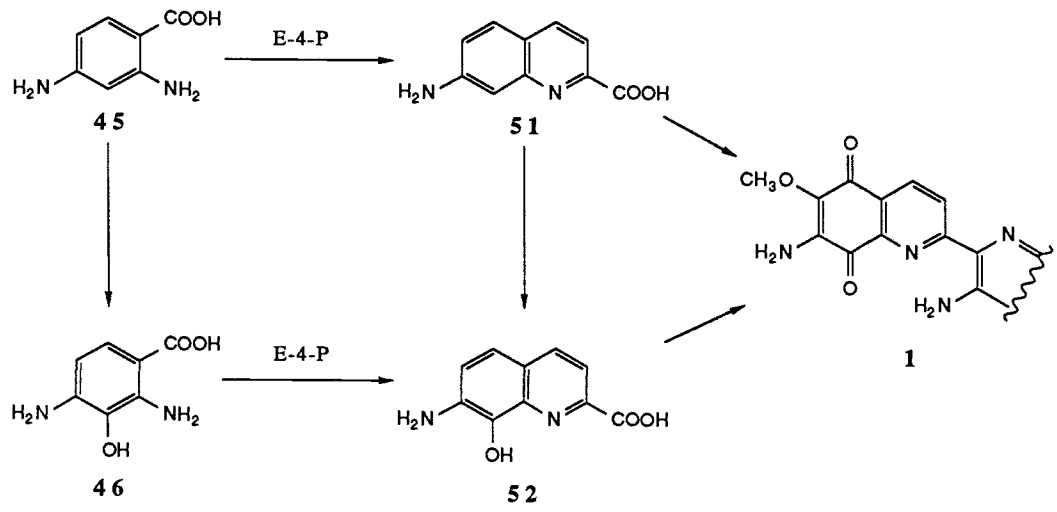
Should this oxidation not have taken place at the anthranilate stage it was quite possible it might have occurred at the quinaldinate stage. Thus the quinoline derivatives **51** and **52** were also synthetic and biosynthetic targets. A grid representing all the permutations is shown in Scheme 6. It was highly unlikely that all of these compounds were actual intermediates unless a metabolic grid existed. Determination of the operative pathway was our main goal.



Scheme 4. Possible Biosynthetic Pathways To Streptonigrin



Scheme 5. Possible Biosyntheses of *m*-Aminoanthranilic Acid and 2,4-Diamino-3-hydroxybenzoic Acid



Scheme 6. Possible Biosyntheses of the Quinoline Quinone Portion of STN

Results and Discussion

Oxygen 16 Fermentations

In an attempt to determine the origin of the numerous oxygen atoms in streptonigrin a fermentation utilizing $^{18}O_2$ enriched gas was planned. Incorporation can usually be determined by analysis of the ^{13}C NMR spectrum of the antibiotic. If an oxygen atom were derived from molecular oxygen, then two resonances should be observed for the corresponding carbon atom in the^{13}C NMR spectrum when a strong magnetic field is used. The natural abundance ^{13}C-^{16}O resonance should be observable along with an isotopically upfield shifted ^{13}C-^{18}O resonance. This phenomenon was first observed independently by two research groups[61,62] and has since been used to determine the origin of oxygen atoms in numerous biosynthetic studies[63].

A closed system using four ball joint flasks joined in series as shown in Figure 10 was initially investigated. Under these conditions streptonigrin production was sufficient (22.4 mg) but a total of nearly five liters of oxygen was consumed over a ninety-two hour period (Fig. 11). In order to decrease the dead volume of nearly four liters (for cost efficiency) and optimize oxygen uptake a fermentation bubbler apparatus (Fig. 12) was built. This design was based on personal communication with Dr. J. C. Vederas of the University of Alberta. Trial operations with autoclaved but uninoculated Pfizer medium gave extensive foaming within fifteen minutes. Should this foaming have continued unabated it would surely have resulted in damage to the aquarium pump. Addition of anti-foaming agents proved fruitless. Concentrations ranging from 0.1 to 0.5% n-butanol, Tween 60™, and Ucon 2000™ (a polyalkylene glycol lubricant) were employed with no significant change. Other unsuccessful tactics included fluctuation of the gas flow rate (from 2 L/min. to 0.5 L/min.) and a twenty-four hour "pre-incubation" period in the conventional Erlenmeyer flasks prior to transfer to the Vederas apparatus. Alternative chemically defined media (shown below) were prepared and tested but to no avail. Anti-foaming agents were also tried when foaming occurred with these, as well.

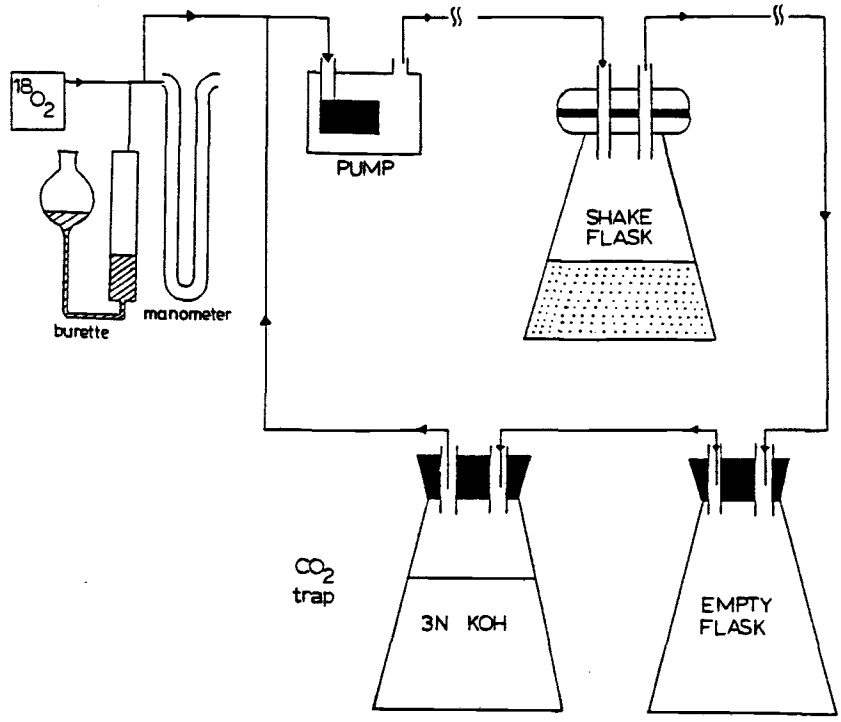


Figure 10. Ball Joint Fermentation Layout

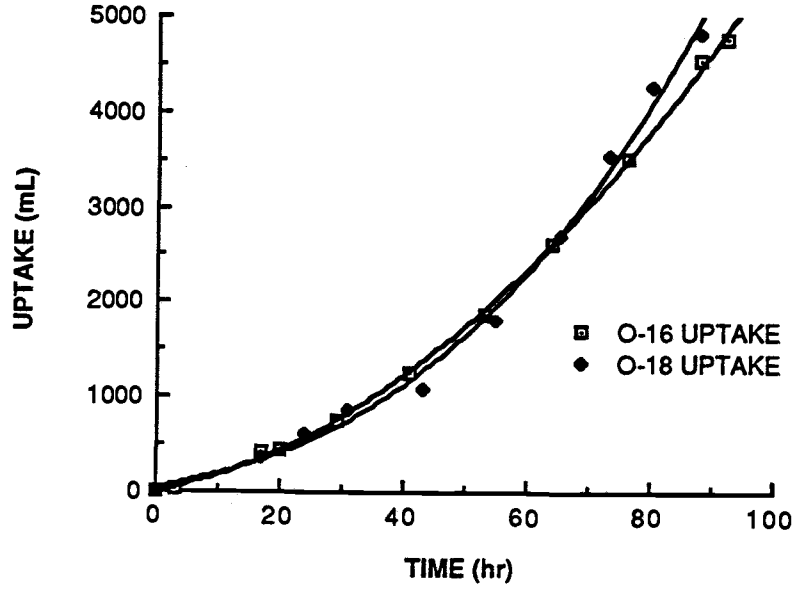


Figure 11. Oxygen Consumption During STN Fermentations

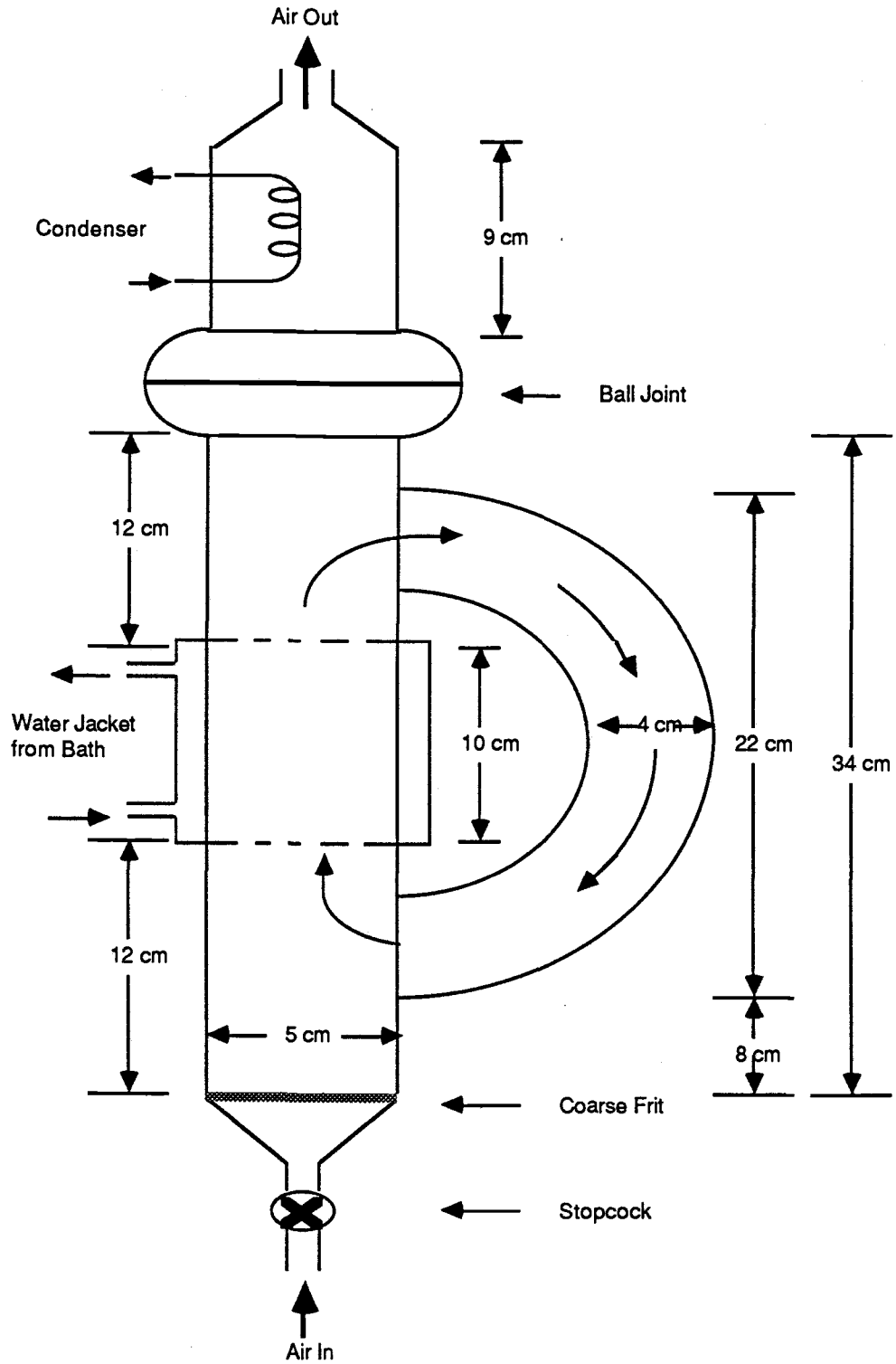


Figure 12. Vederas Apparatus

Fonouni Medium F[64]

glucose (3%), ammonium tartrate (1%), NaCl (0.5%), $NaNO_3$ (0.2%), K_2HPO_4 (0.1%), KCl (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), $CaSO_4 \cdot 2H_2O$ (0.04%), $FeSO_4 \cdot 7H_2O$ (0.001%) plus 10 ml of a liter solution consisting of the following amino acids: arg•HCl, glu, his•HCl, ile, leu, thr, val, ala, asp, gly, ser, cys, trp. All acids were L-isomer except asp which was racemic. All quantities were 300 mg except glycine which was 350 mg.

Result: no antibiotic production

Fonouni Medium I[64]

glucose (1.0%), NH_4Cl (0.5%), KH_2PO_4 (0.05%), K_2HPO_4 (1.5%), Na_2SO_4 (0.5%), $MgCl_2 \cdot 6H_2O$ (0.01%), 25 mL of the amino acid solution used in above medium.

Result: no foaming but low antibiotic production (11 mg)

Emerson Meduim[50]

beef extract	4.0 g
gelysate™ peptone	4.0 g
yeast extract	1.0 g
NaCl	2.5 g
dextrose	10.0 g
water	1.0 L

Result: extensive foaming

In view of these discouraging results we returned to the original ball joint flask method.

Oxygen 18 Fermentation

A fermentation using the original ball joint flask method was performed[65]. For the initial twenty four hours and final twelve hours $^{16}O_2$ gas was used to minimize waste of $^{18}O_2$ gas. Standard work-up after 92 hours afforded 21.1 mg of antibiotic. Analysis of the 100 MHz carbon spectrum on 10 mg of material revealed upfield shifts for the following peaks:

PPM	POSITION
175.84	C-5
153.02	C-10'
147.99	C-8'
136.87	C-9'
135.65	C-6
60.22	C-10' OMe
59.61	C-9' OMe
55.58	C-6 OMe

Relevant portions of the spectrum along with the carboxylate resonance as an unaffected reference are shown in Figure 13.

Several important findings on the biosynthesis of streptonigrin resulted from this experiment. Of particular interest was the mechanism of oxidation of the D-ring and ultimate carboline ring cleavage. Initial oxidative hypotheses[49a] had the C-9' and C-10' oxygens being derived from molecular oxygen. The crucial difference was the source of the C-8' oxygen which could be derived either from oxygen (Pathway A) or from water (Pathway B) as shown in Scheme 7. A third mechanism, which is formally a hydrolysis, was subsequently proposed by another research group[11](Scheme 8); again the C-8' oxygen would have come from water. Clearly this "covalent hydration"[66] theory and Pathway B were incorrect as this experiment showed molecular oxygen was the source of the hydroxyl moiety. Only Pathway A is consistent with this result.

Further exciting results regarding the A-ring were also obtained by this experiment. As expected, the C-6 methoxyl oxygen was derived from molecular oxygen. The source of the quinone carbonyl oxygens were not so readily predicted. An upfield shift at ca. 176 ppm revealed the C-5 carbonyl was derived from O_2. However no such shift could be observed for the C-8 carbonyl. This implied that this oxygen was retained from C-1 of erythrose and made the hydroxylated amino acids **46** and **52** plausible intermediates.

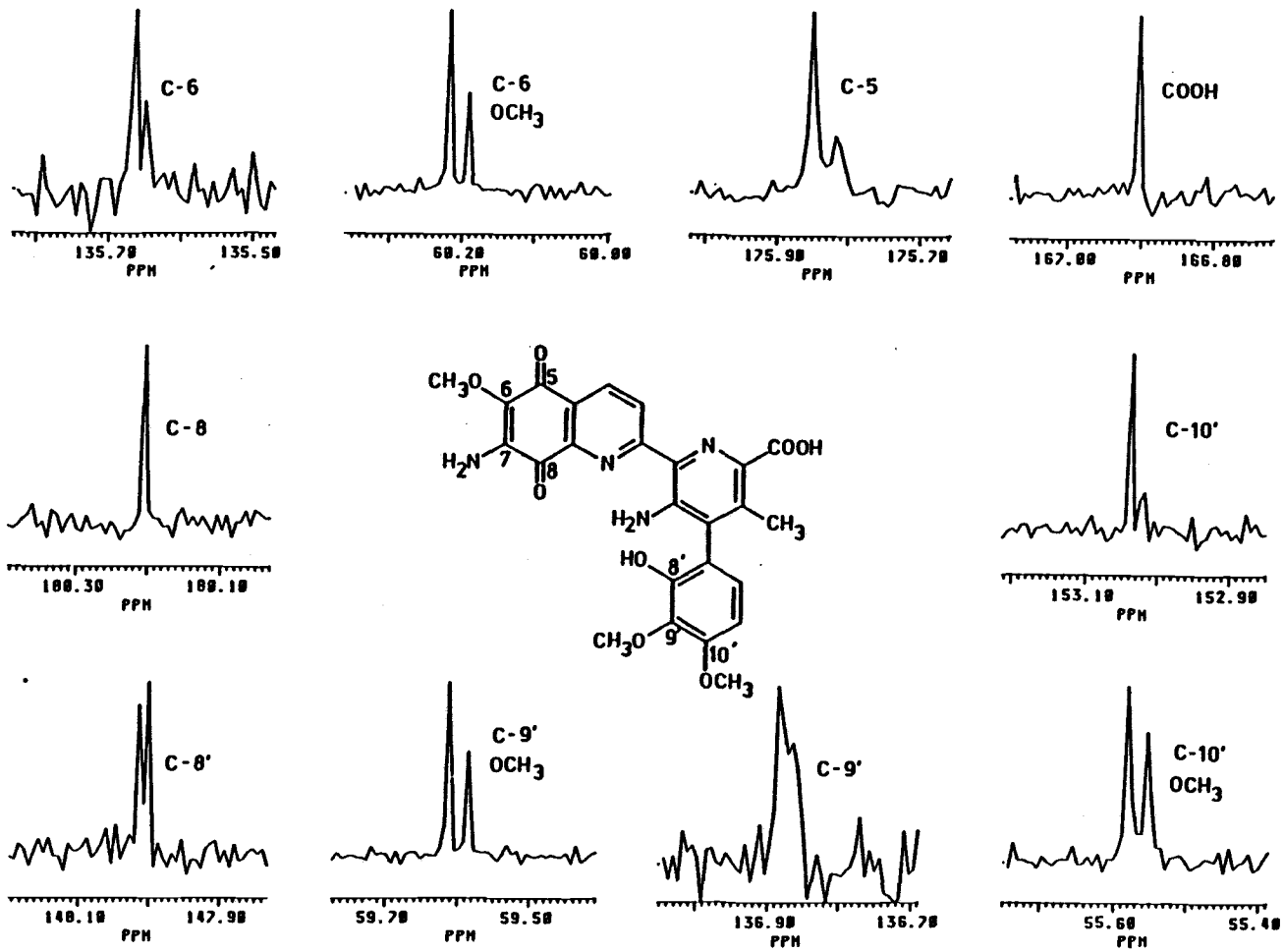
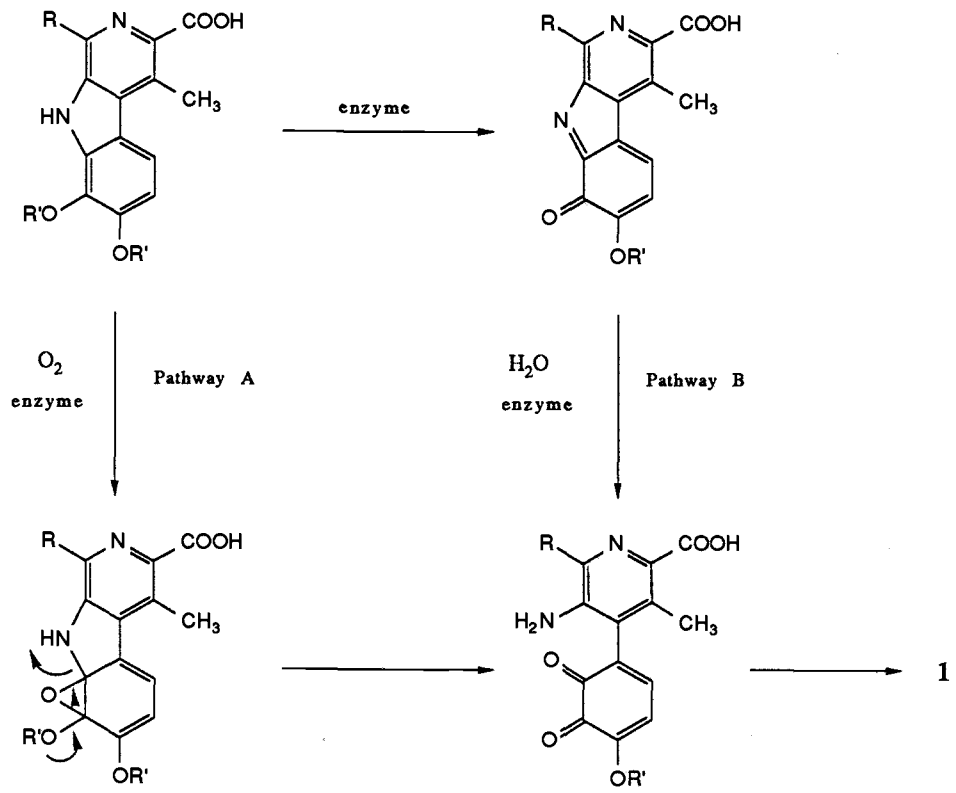
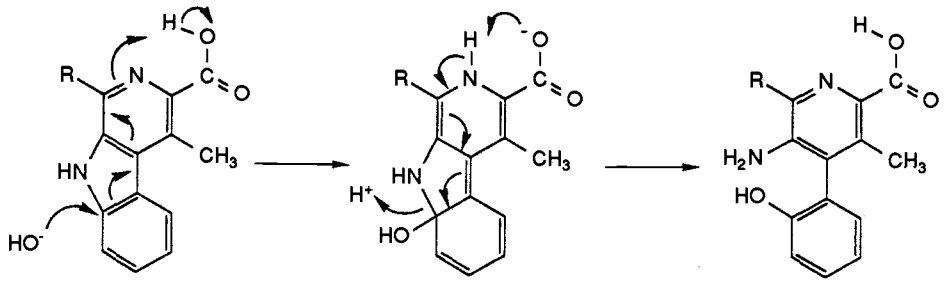


Figure 13. Partial 100 MHz ^{13}C NMR Spectrum of Streptonigrin, **1a**, Produced Under An $^{18}O_2$ Enriched Atmosphere



Scheme 7. Proposed[49a] Oxidative Mechanisms Of β-Carboline Cleavage



Scheme 8. Proposed[11] Hydrolytic Mechanism Of β-Carboline Cleavage

O-18 Water Exchange

Recognizing that the C-8 quinone carbonyl of streptonigrin is part of a vinylogous ester system, we thought that it might be particularly susceptible to exchange with solvent. This could provide an alternative explanation for the apparent lack of incorporation of oxygen from $^{18}O_2$ at this position. Internal hydrogen bonding with the adjacent C-7 amine would also favor formation of a hydrated species. Partial exchange in ketone carbonyls via such hydrated species is well documented[63b]. Studies have been carried out which showed a correlation between the extent of ketone exchange and the pH of the system[67,68]; however, not much literature exists on exchange in quinone carbonyls. The simplest quinone, p-benzoquinone, undergoes 8% exchange[67] with $H_2{}^{18}O$ in one minute at 65° C. During a study of menaquinone biosynthesis complete loss of O-18 label (originally derived from molecular oxygen) occurred during alkaline extractive work-up[69].

To examine the possibility that the carbonyl at C-8 may have undergone solvent exchange, a sample of authentic streptonigrin (20.3 mg, 40.0 μmole) was stirred for ten hours in the dark in a solution of THF and $H_2{}^{18}O$. The use of tetrahydofuran as cosolvent was necessary due to the insolubility STN in water. After isolation by extractive work-up, an NMR analysis on 10 mg of the sample showed no isotopically induced upfield shifts. A portion of authentic STN (10 mg) was admixed and another spectrum acquired but again no shifts were seen. Therefore exchange via a hydrated form did not occur at neutral pH. Alkaline and acidic pH ranges were tested in similar fashion. A sample of STN was stirred overnight in a solution of $THF/H_2{}^{18}O/NaHCO_3$ (pH 10.5). Analysis of the ^{13}C NMR spectrum showed a resonance at 180.20 ppm, upfield from the ^{13}C-^{16}O shift at 180.26 ppm (Fig. 14). The peak height ratio indicated ca. 30% exchange. No such shift was observed for the C-5 carbonyl at 175.9 ppm. An upfield shift at 181.27 ppm from 181.31 ppm (exchange of ca. 15%) was observed when the experiment was performed under acidic conditions (pH 5). Again no such shift was seen for the C-5 carbonyl. Similar quinone exchanges have also been observed in these laboratories for sarubicin, **48**[58], and naphthyridinomycin, **54**[70] (Fig. 15). We therefore felt it reasonable to postulate that the C-8 quinone carbonyl was quite likely derived from molecular oxygen but was subsequently lost either via exchange with the fermentation broth or during the extractive work-up.

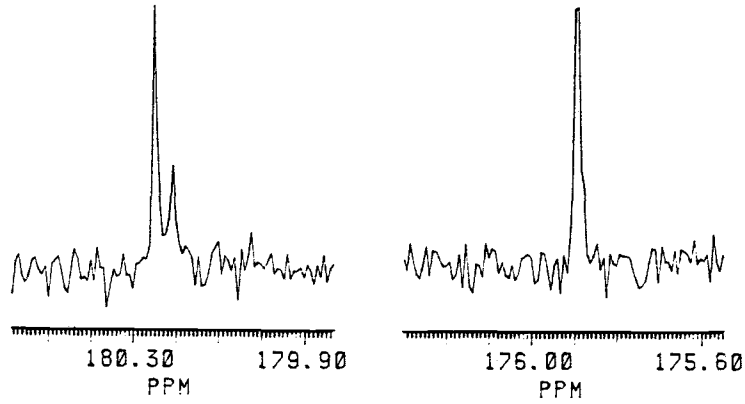
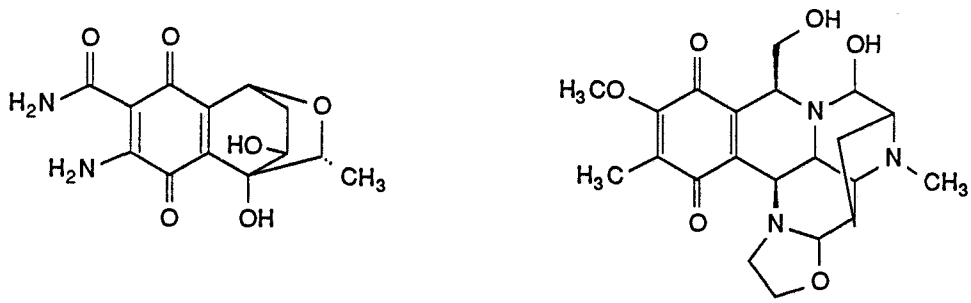


Figure 14. Quinone Carbonyl Resonances of Streptonigrin From O-18 Water Exchange At Alkaline pH



48 54

Figure 15. Examples of Water Exchangeable Quinone Natural Products

Spectral Studies on Streptonigrin and "Sanguinomycin"

In the course of our biosynthetic studies on STN we were in need of a suitable NMR solvent in which proton and carbon resonances were clearly resolved and unobscured by solvent peaks. Deuterated trifluoracetic acid proved to be an ideal candidate and further provided us with spectral information which led to minor revisions of the original spectral assignments of streptonigrin. Using this solvent, the upfield doublet at δ 7.02 in the proton spectrum had slowly disappeared and the downfield doublet at δ 7.07 collapsed to a singlet over a seven-day period. This upfield resonance was therefore attributed to the H-11' proton, para to the free hydroxyl at C-8'.

The downfield resonance was necessarily assigned to H-12'. It was determined that the same relationship existed in dimethyl sulfoxide and that these resonances were inadvertently reversed when initally reported [43]. The proton resonances in TFA-d_1 are shown in Table 2.

During earlier biosynthetic studies in this laboratory it was discovered that a compound of structure similar to **1** was produced along with **1** by *S. flocculus*. This minor co-metabolite was given the trivial name of sanguinomycin and was determined to be lacking an *O*-methyl group at either the C-9' or C-10' position[71]. Based on preliminary NMR studies it was tentatively assigned the structure 10'-*O*-demethylstreptonigrin. (Later this structure would be assigned to a co-metabolite of STN produced by *S.albus*[9]). We felt that a two dimensional NMR study would allow us to unequivocally determine which position the free phenol moiety occupied. Specifically, a two dimensional heteronuclear (^{13}C-^{1}H) long range correlation experiment (LR HETCOSY)[72] was planned. This type of spectrum typically reveals long range coupling through two or more bonds.

To test the feasibility of this approach the spectrum of streptonigrin was first acquired using DMSO-d_6 as solvent. Analysis of the long range couplings between carbon and hydrogen confirmed previous assignments and allowed for the first time definitive assignment of each methoxyl resonance in the proton and carbon spectra. The peak assignments are listed in Tables 3 and 4 and their coupling patterns (3 bond) are listed in Table 5. Expected couplings which are not observed are listed in Table 6. The spectrum itself is shown in Figure 16.

Assignment	Shift (Integral, Multiplicity)
H-3	8.91 (1H, d, J = 8.2 Hz)
H-4	8.66 (1H, d, J = 8.2 Hz)
H-12'	7.06 (1H, d, J = 8.6 Hz)
H-11'	7.02 (1H, d, J = 8.6 Hz)
C-6 OMe	4.25 (3H, s)
C-9' OMe	4.15 (3H, s)
C-10' OMe	4.18 (3H, s)
C-3' Me	2.70 (3H, s)

Table 2. Proton NMR Assignments of Streptonigrin in TFA-d_1

Assignment	Shift (Integral, Multiplicity)
H-3	9.01 (1H, d, J = 8.4 Hz)
H-4	8.36 (1H, d, J = 8.4 Hz)
H-12'	6.73 (1H, d, J = 10.1 Hz)
H-11'	6.71 (1H, d, J = 10.1 Hz)
C-10' OMe	3.86 (3H, s)
C-6 OMe	3.82 (3H, s)
C-9' OMe	3.76 (3H, s)
C-3' Me	2.18 (3H, s)

Table 3. Proton NMR Assignments of Streptonigrin in DMSO-d_6

Assignment	Shift
C-8	180.90
C-5	176.54
COOH	167.61
C-2	160.46
C-10'	153.73
C-8'	148.70
C-5'	146.31
C-8a	144.74
C-7	142.15
C-9'	137.58
C-3	136.77
C-6	136.36
C-2' or C-6'	135.17
C-4'	134.58
C-4	133.96
C-6' or C-2'	130.15
C-4a	127.32
C-3	126.56
C-12'	125.17
C-7'	115.48
C-11'	105.04
C-9' OMe	60.92
C-6 OMe	60.31
C-10' OMe	56.29
C-3' C-Me	17.57

Table 4. Carbon NMR Assignments of Streptonigrin in DMSO-d_6

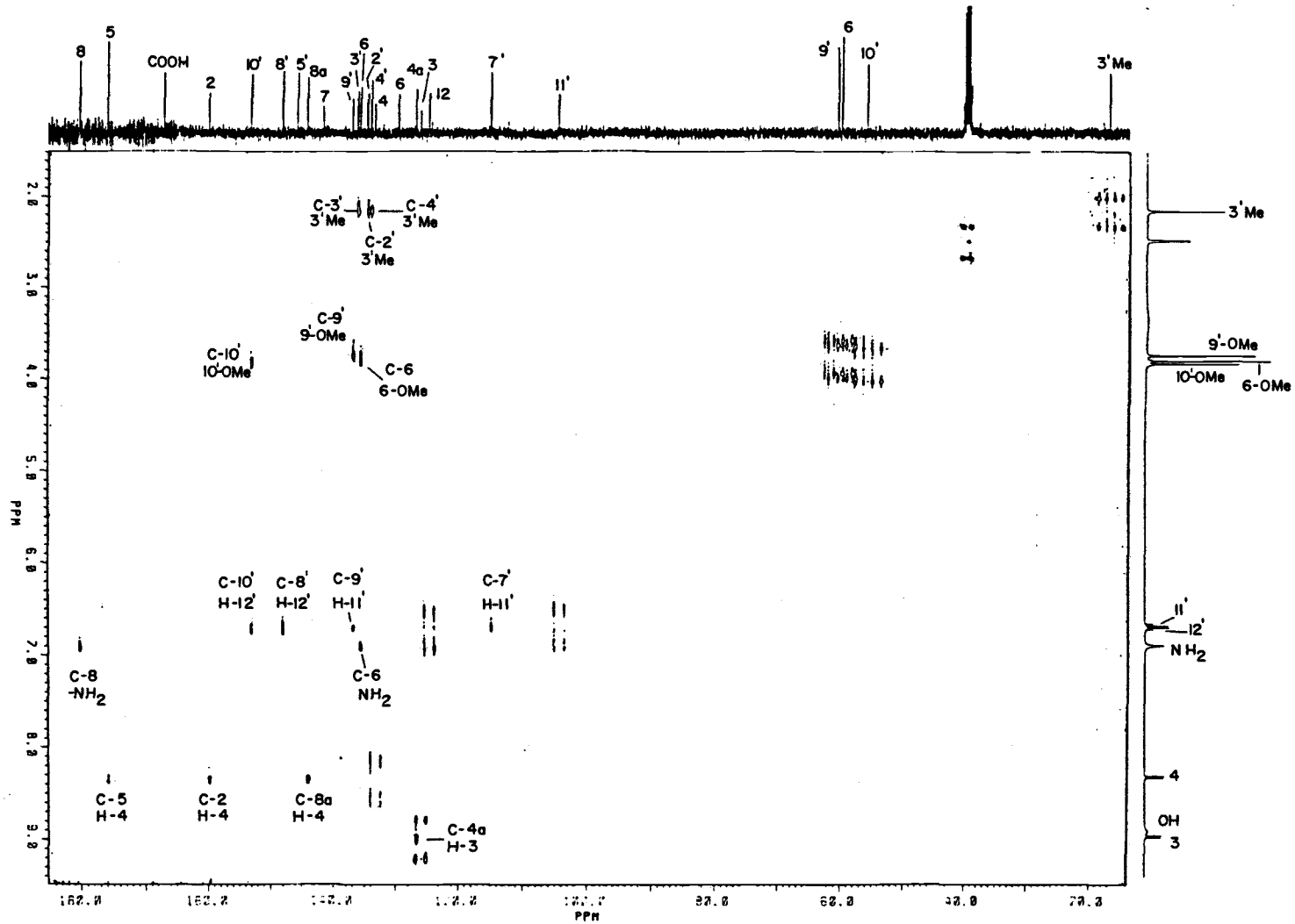


Figure 16. [^{13}C-^{1}H] LR HETCOSY NMR Spectrum of Streptonigrin Acquired in DMSO-d_6

Carbon #	Long Range Coupled Protons
C-2	H-4
C-3	none (direct to H-3)
C-4	none (direct to H-4)
C-4a	H-3
C-5	H-4
C-6	6-OMe, 7-NH_2
C-7	none
C-8	7-NH_2
C-8a	H-4
C-2'	3'-Me
C-3'	3'-Me (2 bond)
C-3' Me	none (direct to Me)
C-4'	3'-Me
C-5'	none
C-6'	none
C-7'	H-11'
C-8'	H-12'
C-9'	H-11', 9'-OMe
C-10'	H-12', 10'-OMe
C-11'	none (direct to H-11')
C-12'	none (direct to H-12')

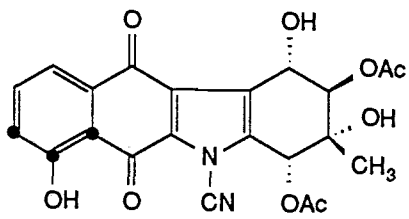
Proton #	Long Range Coupled Carbons
H-3	C-4a
H-4	C-2, C-5, C-8a
H-11'	C-7', C-9'
H-12'	C-8', C-10'
7'-NH_2	C-6, C-8
6-OMe	C-6
9'-OMe	C-9'
10'-OMe	C-10'
3'-Me	C-2', C-3', C-4'

Table 5. Analysis of [^{13}C-^{1}H] LR HETCOSY NMR spectrum of STN in DMSO-d_6

C-4' to H-12'
C-4' to 5'-NH_2
C-6' to H-3
C-6' to 5'-NH_2
C-7' to 8'-OH
C-9' to 8'-OH

Table 6. Expected Couplings That Were Not Observed
In The [^{13}C-^{1}H] LR HETCOSY Spectrum of STN (DMSO-d_6)

While this spectrum revealed numerous long range couplings several were not observed which would have made structure assignment of sanguinomycin trivial. It was initially hoped that coupling between the phenol proton and C-7', C-8', and C-9' of STN could be observed. These types of couplings have been observed in kinamycin, **55**[72], and murayaquinone, **56**[73], and greatly aided in their structural elucidation.



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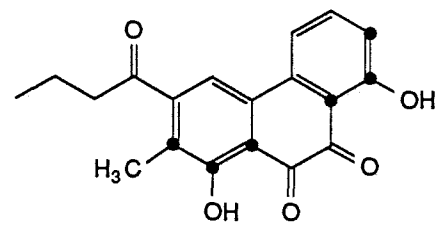

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An alternative approach of "walking around the molecule" employing these long range couplings was also thwarted by lack of coupling between C-4' of the C-ring and H-12' of the D-ring. C-4' could have been unequivocally assigned because it would have been the only carbon peak that showed coupling to the C-3' methyl protons and to an aromatic proton. Furthermore this proton would have had to be H-12'. From there the H-12' resonance could have been used to assign C-10', and C-10' examined to see whether or not it was coupled to methoxy protons. This "hopscotch" approach seemed somewhat roundabout but would have been unequivocal.

A third approach, therefore, held the most promise for success. Earlier in this section the spectrum of STN in TFA-d_1 was reported. In this spectrum the H-11' proton slowly underwent exchange and the H-12' collapsed to a singlet. Expounding to the desmethyl case, if the methyl were missing from the C-9' oxygen then both H-11' and H-12' should have disappeared through exchange as both would be para to free phenolic residues. If the C-10' oxygen were lacking the methyl group then only the H-11' proton would have disappeared. The first scenario would have been unequivocal and further confirmation would not be essential. The second situation would be a "negative result", however, and would have required continued effort.

Long range $^{1}H/^{13}C$ spectra on both unexchanged and exchanged sanguinomycin and comparison of the spectra would have afforded unequivocal assignment. The disappearance of the three-bond coupling of H-11' (now fully deuterated) to C-7' and C-9' would have permitted assignment of these carbons. C-9' would have shown coupling to its methoxy protons and therefore C-10' would have possessed the free phenol. Confirmation would be achieved by the observation of coupling from C-10' to H-12' (now a singlet) and the absence of coupling to methoxy protons. To test this hypothesis a HET-COSY spectrum was acquired on streptonigrin using TFA-d_1 as solvent (Figure 17). Interpretation of this spectrum allowed assignment of the majority of the carbon resonances and these are shown in Table 7. Analysis of spectrum also showed the identical coupling patterns as seen previously in DMSO-d_6. As expected the couplings involving H-11' disappeared upon exchange.

Unfortunately, the above methodology was never applied to sanguinomycin, **3**, as the isolated material on hand could not be sufficiently purified for spectral analyses.

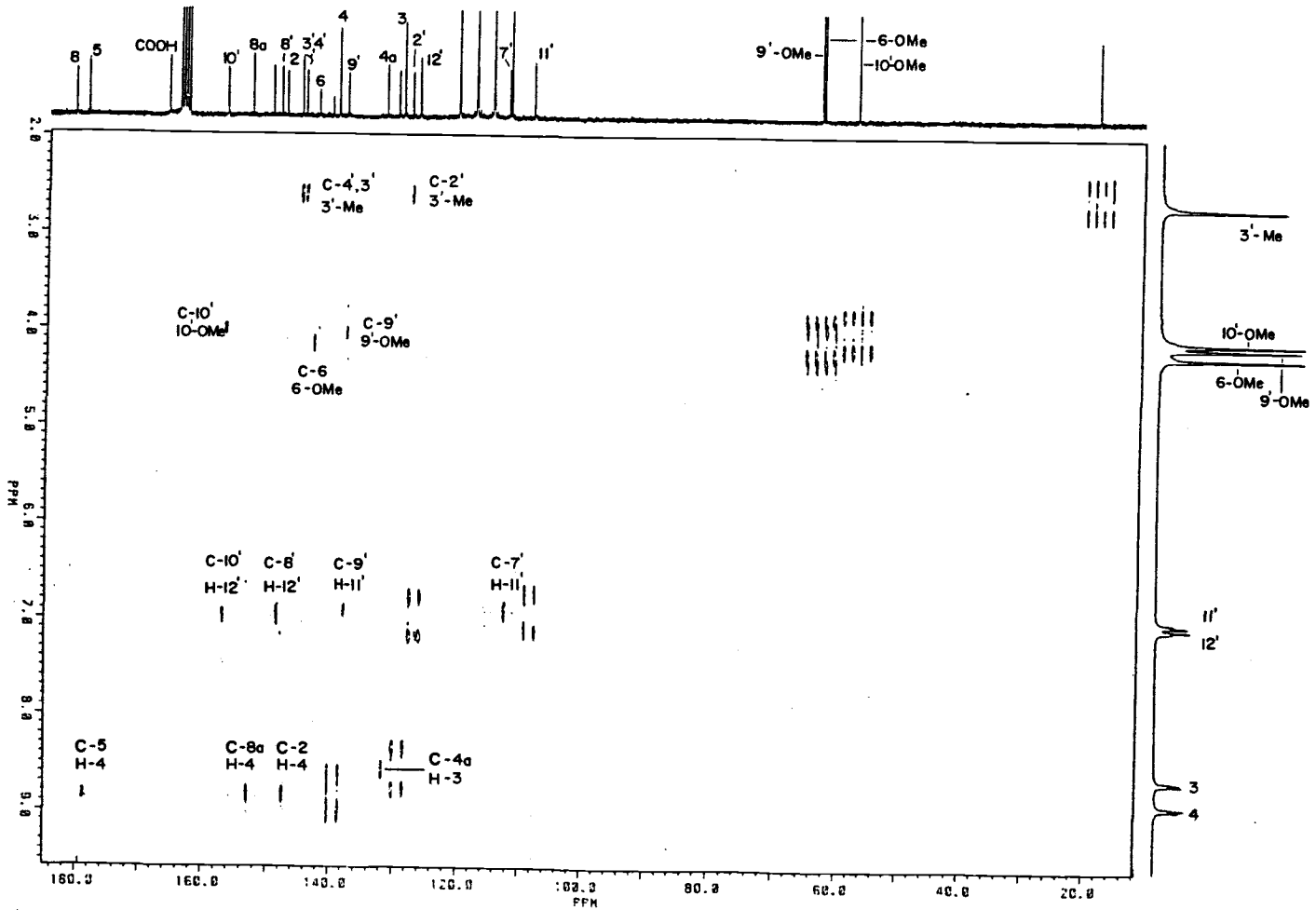


Figure 17. [^{13}C-^{1}H] LR HETCOSY NMR Spectrum of Streptonigrin Acquired in TFA-d_1

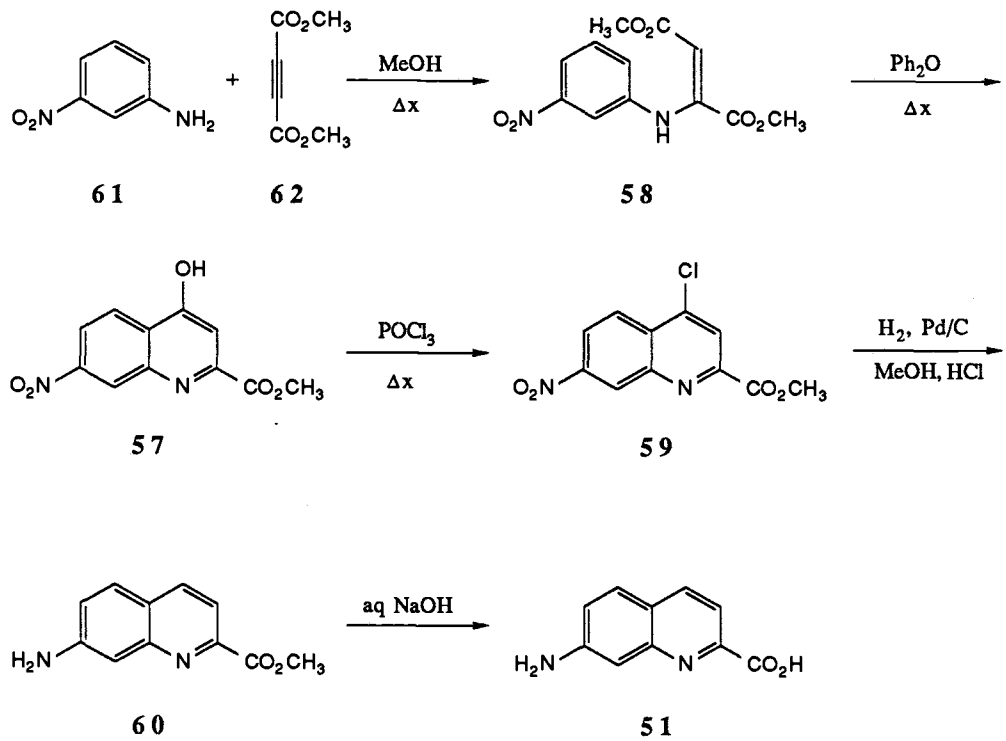
Assignment	Shift
C-8	181.1
C-5	179.0
COOH	166.3
C-10'	157.1
C-2 or C-8a	153.2
unassignable (probably C-5')	150.0
C-8'	148.7
C-2 or C-8a	147.8
C-3' or C-4'	145.4
C-4' or C-3'	144.7
C-6	142.7
unassignable (probably C-7)	140.6
C-4	139.6
C-9'	138.2
C-4a	132.1
unassignable (probably C-6')	130.3
C-3	129.5
C-2'	128.2
C-12'	127.1
C-7'	112.7
C-11'	108.7
C-9' OMe	63.4
C-6 OMe	63.1
C-10' OMe	57.5
C-3' Me	18.6

Table 7. Carbon Assignments of STN in TFA-d_1

Synthesis of Target Compounds

7-Aminoquinaldinic Acid **51**

At the onset of my research on the biosynthesis of streptonigrin, synthesis of the desired 7-aminoquinoline-2-carboxylic acid **51** was envisioned as proceeding from the known methyl 7-nitro-4(1H)-quinolone-2-carboxylate **57**[74] (Scheme 9). This bicyclic compound was reportedly available via cyclization of the fumarate adduct **58**. Chlorination via well documented procedures[75,76,77] would afford the adduct **59**, which after dual catalytic reductions would afford the amino methyl ester **60**. Saponification would then afford the desired target compound. The efficacy of this route was two-fold as a deuterium label could be introduced at the C-4 position by employing deuterium gas as reductant to afford **51a**. This label would have been ideal because, should incorporation have proved positive, it would have been readily detectable by ^2H NMR. Loss of label after incorporation would have been highly unlikely as the C-4 position of streptonigrin is an unexchangeable position.



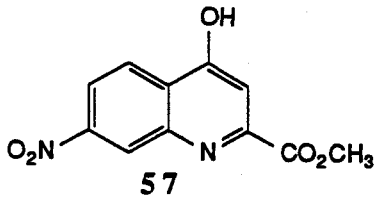
Scheme 9. Proposed Synthesis of 7-Aminoquinaldinic Acid **51**

In practice this synthesis proved much more challenging than initially expected. In accord with the literature procedure, condensation of *m*-nitroaniline **61** with dimethyl acetylenedicarboxylate, (DMAD) **62**, gave the known fumarate adduct **58**[74] without event in 79% yield. Subsequent cyclization in boiling diphenyl ether afforded a fluffy yellow adduct which proved very difficult to handle due to its static charge. Contrary to literature this product was found to be virtually insoluble in methanol as well as a variety of other solvents. Also contrary to literature the ^1H NMR (TFA-d_1) showed the presence of two products. It was claimed that only the 7-nitro adduct was formed:

> " In trifluoroacetic acid solvent the unsplit methyl appeared at 3.82 ppm, the singlet proton on C-3 at 7.67 ppm, the singlet C-8 proton at 8.75 ppm, and an AB quartet centered at 8.27 ppm for the mutually coupled (J = 9 cps) C-5 and C-6 protons."

After tedious repetitive recrystallizations from large volumes of methanol a separation of both products was finally achieved. The compound having the higher R_f value (0.53 on aluminum backed silica plates, 6 $CHCl_3$: 1 MeOH) was believed to be the 7-nitro isomer, **57**, by the presence of a downfield singlet in the ^1H NMR spectrum, although its melting point (> 300 °C) varied tremendously from the reported value (268-9 °C). The lower R_f compound (0.45) was determined to be the 5-nitro isomer, **63**, by interpretation of ^1H NMR. Its melting point was 263-4 °C. Each of these isomers was converted to its methyl aminoquinoline-2-carboxylate via the sequence shown in Scheme 9. The crude intermediates of each isomer were analyzed by ^1H NMR which confirmed the assignment of each isomer. These assignments are shown in Figure 18.

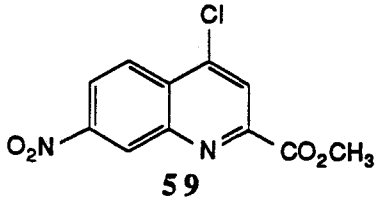
While the above reactions were undertaken an alternative route toward the methyl 7-nitro-(4H)-quinolone-2-carboxylate was briefly examined. It was theorized that an *ortho* carboxyl substituent on the aniline ring would direct cyclization toward that position and, after decarboxylation, would afford the desired quinolone isomer as the sole product (Scheme 10). Thus condensation of *m*-nitroanthranilic acid, **64**, with **62** afforded the fumarate adduct **65** in roughly 50% yield. Cyclization in diphenyl ether at



57

Solvent: TFA-d_1

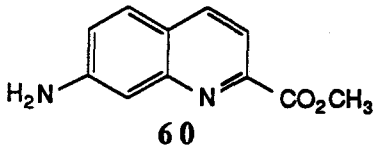
4.35 (3H, s)
8.15 (1H, s), H-3
8.65 (1H, dd, J = 9, 2 Hz), H-5
8.85 (1H, dd, J = 9, 1Hz), H-6
9.25 (1H, br s), H-8



59

Solvent: $CDCl_3$

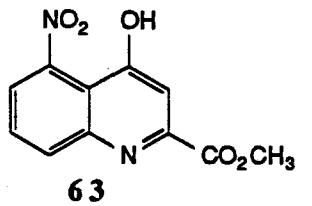
4.10 (3H, s)
8.40 (1H, s), H-3
8.45 (2H, m), H-5, H-6
9.20 (1H, dd, J = 2, 1 Hz), H-8



60

Solvent: DMSO-d_6

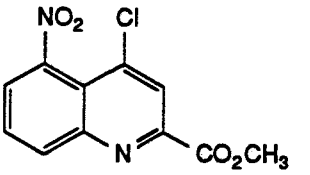
3.90 (3H, s)
7.05 (1H, br s), H-8
7.15 (1H, dd, J = 9, 2 Hz), H-6
7.65 (1H, d, J = 8 Hz), H-4
7.75 (1H, d, J = 9 Hz), H-5
8.30 (1H, d, J = 8 Hz), H-3



63

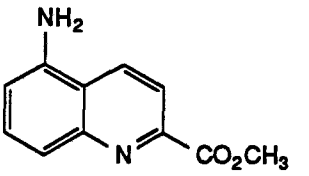
Solvent: TFA-d_1

4.30 (3H, s)
8.05 (1H,dd, J = 11, 2 Hz), H-8
8.10 (1H, s), H-3
8.30 (1H, dd, J = 11, 9 Hz), H-7
8.60 (1H, dd, J = 9, 2 Hz), H-6



Solvent: $CDCl_3$

4.10 (3H, s)
7.80 (1H, d, J = 5Hz), H-8
7.85 (1H, d, J = 5Hz), H-6
8.35 (1H, s), H-3
8.45 (1H, dd, J = 6, 6 Hz), H-7

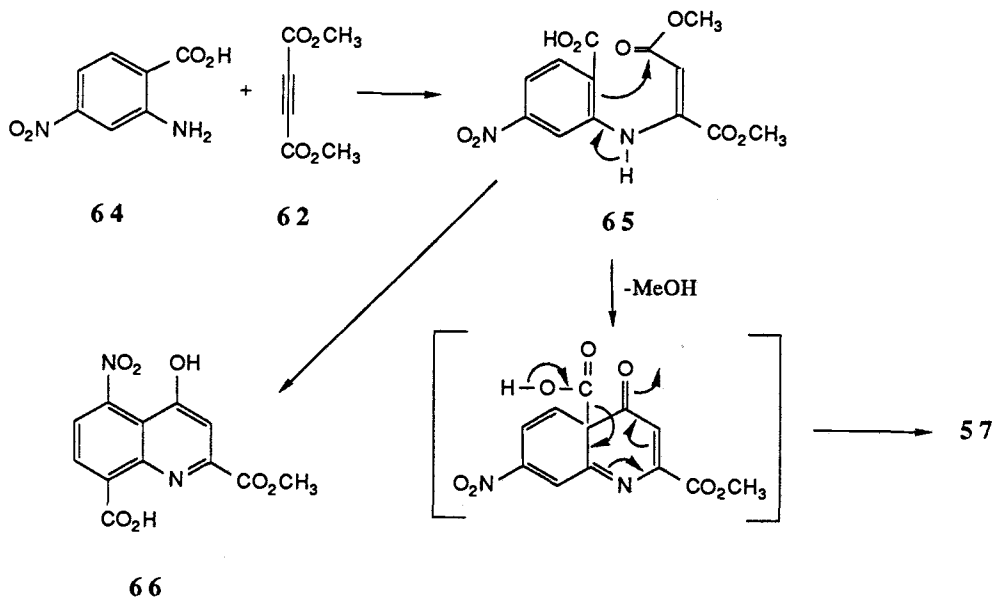


Solvent: DMSO-d_6

3.95 (3H, s)
6.80 (1H, dd, J = 8, 1 Hz), H-6
7.30 (1H, dd, J = 8, 8 Hz), H-7
7.55 (1H, dd, J = 8, 1 Hz), H-8
7.90 (1H, d, J = 9 Hz), H-4
8.70 (1H, d, J = 8 Hz), H-3

Figure 18. Proton NMR Comparison of The 5-Substituted and 7-Substituted Synthetic Intermediates

reflux proceeded smoothly to afford a crystalline product. Unfortunately this product was not the desired **57**. Because the ^{1}H NMR (80 MHz, TFA-d_1) showed only three aromatic protons and the ^{13}C NMR (20 MHz, TFA-d_1) showed two carboxylate resonances (one ester and one acid) the product was tentatively assigned the structure methyl 5-nitro-8-carboxy-(4H)-quinolone-2-carboxylate **66**. Similar products have been reported with cyclizations of methyl anthranilate ester fumarate adducts[78,79].



Scheme 10. Cyclization of *meta*-Nitroanthranilic Acid Adduct **65**

In an effort to facilitate decarboxylation this cyclization was repeated in the presence of 1.1 equiv of sodium bicarbonate. However this led to total decomposition. This carboxylate directing avenue was not pursued further.

Returning to the previously employed methodology, an effort to increase the ratio of cyclization products toward the 7-substituted isomer was examined. A series of reactions utilizing other solvents was undertaken. *ortho*-Dichlorobenzene (bp 180 °C) has been used in similar ring closures of substituted allenes[80] and therefore was tried. In this case the same two products were formed but the dominant isomer was the 5-nitro adduct. Other solvents tried were toluene (bp 110 °C), xylenes (bp 137-44 °C), and nitrobenzene (180 °C). In each case both isomers were formed; however, the

higher the boiling point of the solvent the more 7-nitro adduct was formed. In accordance with this trend triethylene glycol (bp 285 °C) was tried as reaction solvent. In this case rapid massive decomposition occurred.

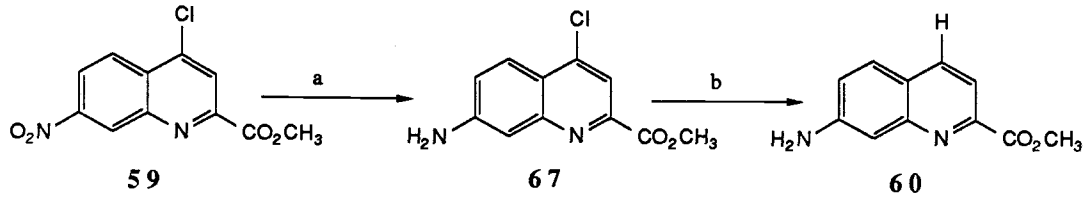
It was obvious at this point that diphenyl ether was the best solvent in which to run the reaction but that a suitable method of separation of isomers needed to be found. After several attempts it was realized that a higher yield could be achieved by adding the crystalline fumarate to the boiling solvent rather than heating a solution of the adduct to reflux temperature. A literature study[81] on cyclizations of various *meta*-substituted anilino fumarates showed a correlation between the solvent/adduct ratio and the 5-substituted/7-substituted product ratio. The more dilute the reaction mixture used the higher percentage of 7-substituted isomer formed. However, increased dilution also led to decreased overall yield. An initial trial at a 30:1 weight ratio followed by separation of isomers by recrystallization from glacial acetic acid[82] (also a new procedure) gave exclusively the desired isomer in 47% yield. A 50:1 ratio afforded the desired compound in a maximum of 68% although yields were normally in the 55-60% range with this solvent ratio. Some results are summarized as shown:

Fumarate (g)	Diphenyl ether (g)	Weight Ratio	Yield
1.0	30	30:1	47%
6.2	270	43:1	56%
13.8	690	50:1	68%

Having finally arrived at a suitable method for synthesis of the desired quinolone we turned our attention to continuation of the synthetic sequence shown in Scheme 9. The chlorination[77] to afford methyl 4-chloro-7-nitroquinoline-2-carboxylate **59** proceeded without incident in 89% yield. The subsequent reductions proved to be a formidable obstacle, however.

The next objective entailed reductive cleavage of chlorine from the quinoline ring and reduction of the nitro group to an amine group. Both of these reactions have been reported for numerous aromatic systems[83] though neither of them simultaneously. When catalytic reduction (H_2 gas, 10% Pd/C, methanolic HCl) was performed on a small scale (ca. 25 mg) the crude product isolated appeared to be solely the desired methyl 7-aminoquinoline-2-carboxylate **60** as determined by ^1H NMR (80MHz, DMSO–d_6). Upon scale up,

however, two main products along with two or three minor products were always detected. Under a variety of conditions tested these same products were always present. It was therefore decided to attempt reduction of the nitro group first and address reductive cleavage of the chlorine afterwards (Scheme 11).



59 **67** **60**

a) $SnCl_2 \cdot H_2O$, concd HCl, 0 °C-25 °C, 4-12 h
b) 1 atm H_2 , 1.3 equiv KOH, 10% Pd/C, MeOH

Scheme 11. Synthesis of Methyl 7-Aminoquinaldinate **60**

Adam's catalyst (PtO_2)[84] gave unpromising results as did employing an acetic acid/sodium acetate buffer[85] in place of methanol. An iron metal reduction in acetic acid/water solution[75] and a sodium dithionite reduction[86] proved to be very slow reactions and yielded multiple products. Finally a satisfactory method was developed using stannous chloride dihydrate in concentrated hydrochloric acid[87]. On small scale reactions this method typically afforded near quantitative yields of methyl 7-amino-4-chloroquinoline-2-carboxylate **67** while multi-gram scale typically afforded 85-90%.

The next question to be addressed was the problem of reductive removal of the chlorine from the C-4 position. Initially it was hoped that the catalytic methods previously tried on **59** would work on the amino chloro adduct. Treatment of **67** with palladium on charcoal in methanol in the presence of hydrochloric acid gave only 10-20% reduction prior to cessation of hydrogen uptake. Even under increased pressures (up to 32 psi) in a Parr shaker apparatus the reaction always stopped prior to completion. Other methods were therefore explored.

A method for halide replacement with deuterium on various aromatic rings (benzenes, indoles, thiophenes) with sodium borodeuteride has precedent[88]. This method would have been particularly fortuitous since we also desired to introduce a deuterium label. Treatment of **67** with two equivalents of palladium chloride and ten equivalents of sodium borohydride in methanol led to total consumption of starting material but gave several products, one of which was the desired **60** as judged by TLC. While this product could be isolated after column chromatography the yield was poor. For this reason, as well as the waste of labelled reagent, this method was deemed not suitable for our needs.

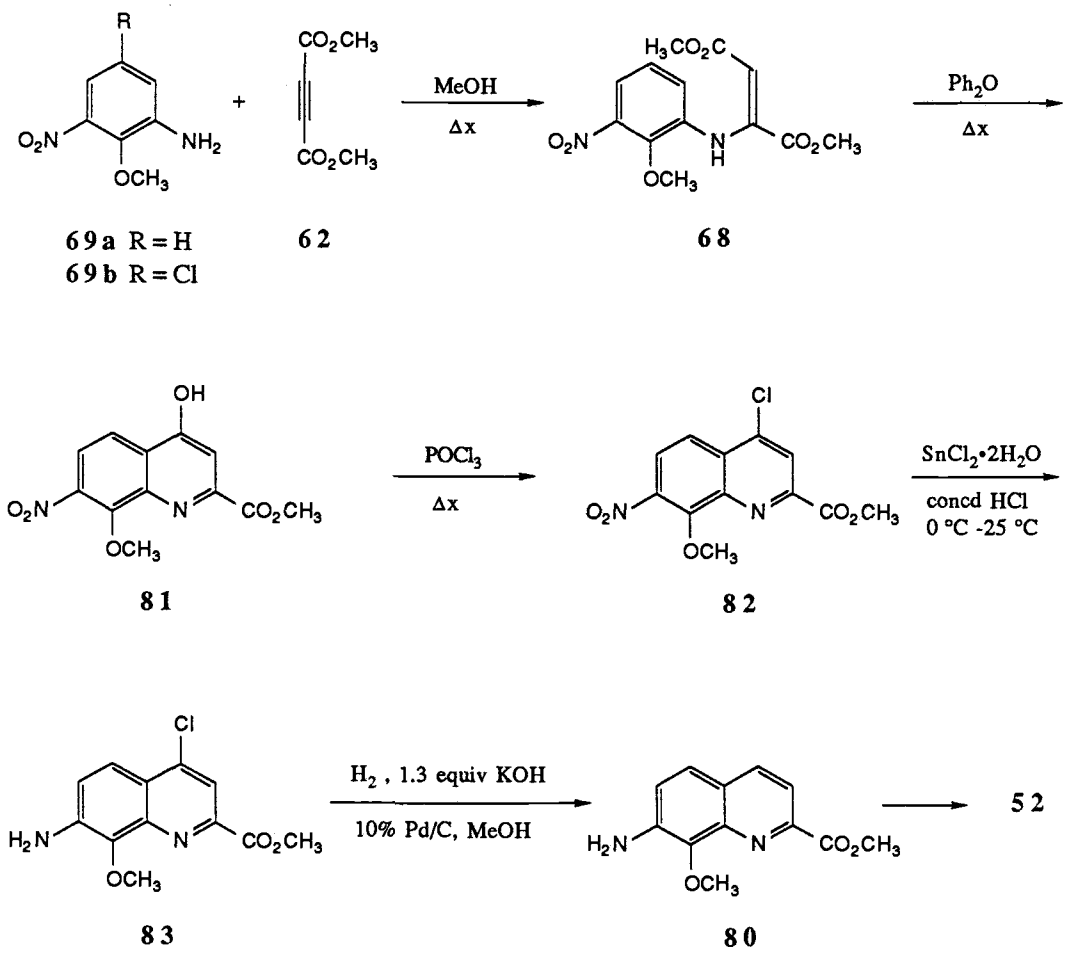
Closer scrutiny of the literature revealed that halide cleavage with palladium catalysts is greatly facilitated by the use of alkaline conditions[89]. Thus when a sample of **67** was hydrogenated using 5% palladium on a calcium carbonate support the reaction proceeded smoothly and quickly to afford **60** as the sole isolable product. Using 5 or 10% palladium on charcoal along with a slight excess of base in the reaction mixture worked just as well.

Having finally acquired the methyl 7-aminoquinoline-2-carboxylate all that remained was simple saponification of the ester to the free carboxylic acid. This was readily achieved by mild reflux in dilute aqueous sodium hydroxide affording the desired 7-aminoquinoline-2-carboxylic acid **51** in crude quantitative yield. Recrystallization from methanol/water afforded **51** in analytically pure form as orange fluffy needles.

The required deuterated analog, [4-^{2}H] 7-aminoquinoline-2-carboxylic acid, **51a**, was synthesized in an analogous fashion using deuterium gas in the reductive cleavage step.

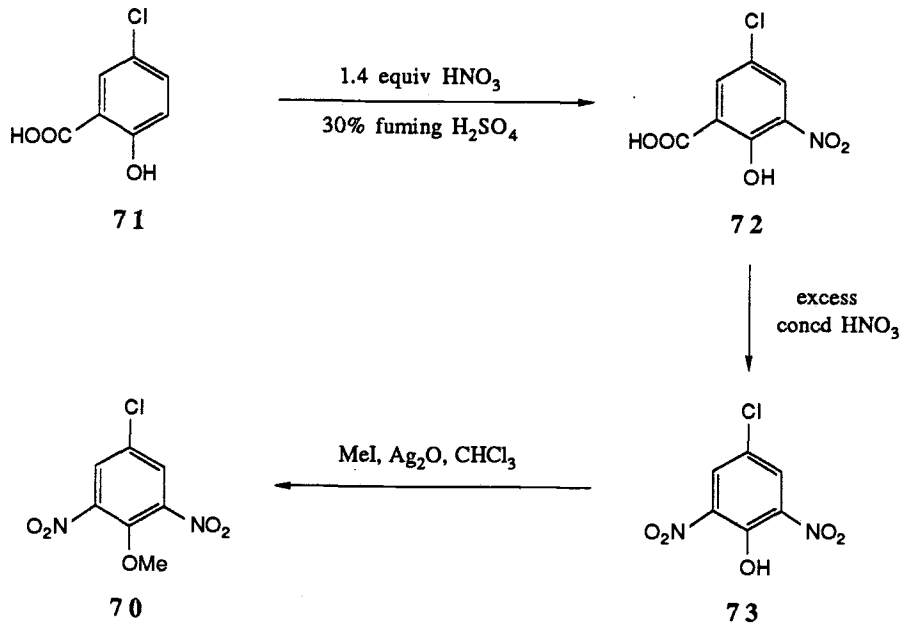
7-Amino-8-hydroxyquinaldinic Acid **52**

Retrosynthetically it appeared wise to adopt the previously used methodologies to the synthesis of the 8-hydroxy analog **52**. Thus the synthesis was envisioned as being identical to **51** after realization of the fumarate adduct **68** as shown in Scheme 12. Therefore a suitably protected synthetic equivalent of 2-hydroxy-3-nitroaniline was required for condensation with **62**. Two possibilities were imagined; the first required 2-amino-6-nitroanisole, **69a**, and the second required 2-amino-4-chloro-6-nitroanisole, **69b**. Due to the inherent difficulty in the syntheses of 1,2,3-trisubstituted benzenes as well as previous literature work and availability of starting materials the latter choice was first addressed. Furthermore, the decision was influenced by the desire to use a chlorinated quinoline intermediate later on in a proposed synthesis of lavendamycin, **4**.



Scheme 12. Proposed Synthesis of 7-Amino-8-hydroxyquinaldinic Acid **52**

It was felt that the amine **69b** could easily be derived from the dinitro compound **70** by partial reduction. Previous syntheses of **70** have been reported[90]. Therefore, following literature procedure[90b,91], the readily available 5-chlorosalicylic acid **71** was converted to **70** by the sequence shown in Scheme 13. Treatment of **71** with 1.4 equivalents of concentrated nitric acid in a fuming sulfuric acid/concentrated sulfuric acid mixture at room temperature afforded 5-chloro-3-nitrosalicylic acid, **72**, in 39% yield. Upon further treatment with nitric acid **72** underwent an ipso substitution to afford 4-chloro-2,6-dinitrophenol, **73**, in low yield. Alternatively **71** was converted directly to **73** in 65% yield by treatment with excess concentrated nitric acid. This phenol was then smoothly transformed into **70** in 70% yield by treatment with methyl iodide and silver oxide in chloroform at reflux[91].



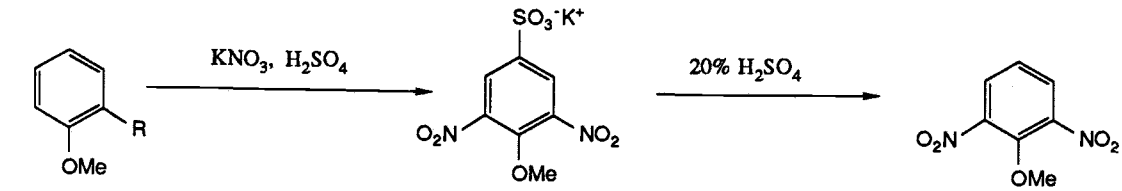
Scheme 13. Synthesis of 4-Chloro-2,6-dinitroanisole **70**

Selective reduction of one nitro group of **70** proved cumbersome. Initially a variety of Zinin reductions (which employ sodium sulfide as

reductant) were examined, none of which showed results that were readily amenable to large scale preparations. Treament of **70** with sodium sulfide in aqueous ammonium chloride at reflux led to partial formation of a product (R_f 0.43, 3:1 hexane : ethyl acetate) believed to be **69b** as judged by its mass spectrum (parent peak at 204 (31.4), base peak at 202 (100)), but it could not be isolated in reasonable fashion. This methodology has been used to obtain 2-amino-6-nitrophenol from 2,6-dinitrophenol[92]. Substitution of methanolic ammonia for water in the previous reaction resulted in a viscous tar. No improvement was seen upon lowering the reaction temperature to 50 °C. In the hope that performing the reaction under acidic conditions would be more successful, concentrated hydrochloric acid was substituted for the methanolic ammonia. Under these conditions, however, the reaction was quite sluggish and was not pursued further.

Discontent with the results of the above route, it was decided to abandon it and examine possible syntheses of 2-amino-6-nitroanisole **69a**. This compound is reportedly available by hydrogen sulfide reduction of 2,6-dinitroanisole **74**[93,94]. Many syntheses of **74** have been reported[93-95], but are low yielding, produce **74** as a mixture with 2,4 dinitroanisole, or are derived from scarce 1,2,3-trisubstituted benzenes. Therefore several approaches were simultaneously examined.

Initial attempts involved nitration of anisole, **75**, and *ortho*-nitroanisole, **76**, which had been protected at the *para* position by a sulfonate group (Scheme 14). However treatment of **76** with fuming sulfuric acid led to a black tar and treatment with concentrated sulfuric acid was ineffective as only starting material was detectable. Attempts using potassium nitrate in sulfuric acid afforded a product which was not characterized but believed to be the sulfonate salt **77**. This procedure has been employed in the synthesis of 2,6-dinitroaniline from chlorobenzene[96]. While vigorous reflux of **77** in 20% sulfuric acid afforded trace amounts of **74**, this route was deemed not feasible for the multi-gram scale necessary. When the identical conditions were tried on **75** similar discouraging results were obtained. Therefore these materials were abandoned as suitable starting materials.



75 R=H
76 R=NO_2

77

74

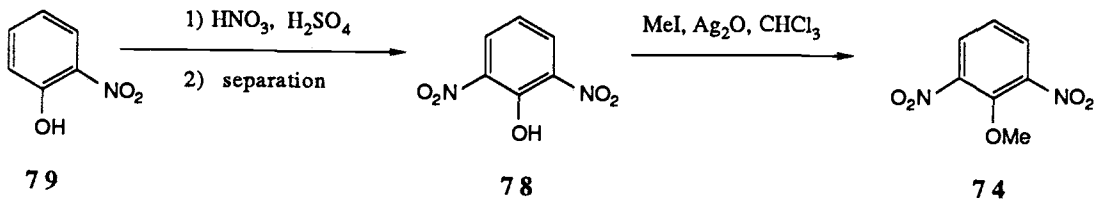
Scheme 14. Attempted Nitration of Anisole **75** and *ortho*-Nitroanisole **76**

Attention was then focused on obtention of 2,6-dinitrophenol, **78**, which could easily be methylated to afford **74**. Diazotization of 2,6-dinitroaniline with sodium nitrite in sulfuric acid[97] or nitrosylsulfuric acid[98] and subsequent quenching of the diazonium salt with water both led to formation of product as detected by TLC. However, in neither case could a product be isolated from the reaction mixture.

Unsatisfied with the previous limited success, **78** was synthesized in multi-gram quantity by a minor modification of a laborious but established procedure[92] as depicted in Scheme 15. *ortho*-Nitrophenol, **79**, was treated with a nitric/acetic/sulfuric acid solution affording a mixture of **78** and 2,4-dinitrophenol. Taking advantage of the lower solubility of 2,6-disubstituted phenoxide barium salts, the desired isomer was then isolated in 20% yield by separation from 2,4-dinitrophenol through a series of barium salt/sodium salt interconversions. Subsequent methylation using methyl iodide and silver oxide in chloroform[90d,91] proceeded smoothly to afford **74** in 88% yield.

Having **74** at hand, attention was again focused on selective reduction of only one nitro group. Unfortunately, this again proved difficult as was the case with the previously discussed chloro adduct **70**. Application of previously cited conditions for the Zinin reduction were ineffective. Additional variations included sodium sulfide and elemental sulfur in methanol at reflux[99] and an aqueous sodium persulfide solution at room temperature[100]. The former was a sluggish reaction and the latter gave no reaction at all. Literature preparations[93,94] employing a stream of

hydrogen sulfide gas bubbling through a methanolic solution of **74** afforded product but only in 20% yield. In my hands this methodology was quite cumbersome and a nuisance for such a low yield. Still other methods were investigated as a cleaner method was desired.



79 **78** **74**

Scheme 15. Synthesis of 2,6-Dinitroanisole **74**

Attempted atmospheric catalytic hydrogenation utilizing 10% palladium on charcoal and dimethoxyethane as solvent gave only partial reduction. This method has been successfully used in the selective reduction of 2,6-dinitroaniline to 1,2-diamino-3-nitrobenzene[101].

Stannous chloride in concentrated hydrochloric acid gave no reaction. Changing to acetic acid as solvent and mild heating (35 °C) led to two minor products but mostly unreacted **74**. An increase in the temperature (85-90 °C) led to total consumption of starting material but no tractable products. This methodology has been utilized in the conversion of 2-chloro-1,3-dinitrobenzene to 1-amino-2-chloro-3-nitrobenzene[102].

A suitable and efficient methodology was finally developed employing in situ generation of hydrogen[103]. Treatment of a triethylamine/acetonitrile solution of **74** with a formic acid/acetonitrile solution in the presence of 10% palladium on charcoal afforded smooth reduction to **69a** plus a small amount of another compound believed to be the diamine analog. Preferential crystallization by digestion in hot hexanes afforded crystalline **69a** in 78% yield. The reaction was easily regulated by monitoring the amount of formic acid solution added.

Having finally obtained the aniline adduct **69a** the remainder of the synthesis proceeded virtually trouble-free up to methyl 7-amino-8-methoxyquinoline-2-carboxylate **80**.

Condensation of **69a** with **62** in methanol at reflux afforded the dimethyl (2'-methoxy-3'-nitroanilino) fumarate, **68**, as yellow needles in an optimum of 84% yield. Typical yields were consistently in the 75-80% range. Cyclization in diphenyl ether afforded the quinolone **81** as the sole product as ring closure could only occur at one site. Yields for this reaction initially were in the 70-75% range but were optimized to the low 90's. Treatment of **81** with an excess of phosphorus oxychloride afforded **82** as a white solid after extractive work-up, decolorization, and purification by flash chromatography. The nitro group was uneventfully reduced in typical fashion to afford the chloro amine **83** in high yield. Reductive removal of chlorine as before afforded **80** in crude quantitative yield. A small amount of ester hydrolysis accompanied this reaction but the acid was easily removed by flash chromatography to give pure **80** in 80-90% yield.

On occasion this reduction would not proceed to completion. The reason for this appeared to be catalyst poisoning by the presence of trace amounts of tin salts carried over from the previous reaction. Several precautions were taken to eliminate this problem when labelled material was prepared or when the reaction was carried out on a large scale. During the isolation, the ethyl acetate solution of the chloro amine adduct **83** was washed with dilute sodium hydroxide. In most cases the crude product was then reduced without difficulty. The use of a larger quantity of catalyst (one-fourth to one-third the mass of substrate as compared to the typical one-tenth) also guaranteed a complete reaction. Purification of crude **83** by flash chromatography also helped but resulted in some loss of starting material.

All that remained was what appeared to be the facile deprotection of the aryl methyl ether and saponification to the free acid. In practice this reaction was erratic, unpredictable, and quite frustrating as no tangible product or derivative thereof was ever isolated.

Many methods of demethylation of aryl methyl ethers exist. A battery of methods was applied, all of which led to either no reaction or erratic production of a variety of unisolable products as shown below. On several

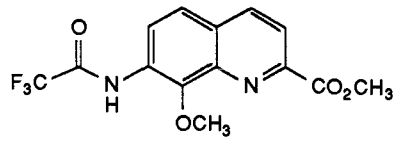
occasions the methoxy acid was formed as judged by TLC. Conditions tried included:

1M BBr_3 in CH_2Cl_2, -80 °C to room temperature[104]
48% HBr in HOAc, Δx[105]
5 equiv NaCN in DMSO, 150 °C[106]
47-51% HI, Δx [94]
3 equiv $AlCl_3$, CH_3CH_2Cl in CH_2Cl_2[107]
1.5 equiv TMSI in sulfolane, 60 °C[108]

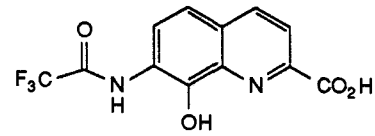
At the time it was thought that decomposition was the major event as no tractable product could be detected. In hindsight, however, the putative hydroxy acid **52** was probably formed as an unstable species in the majority of cases. In none of the cases was a product reliably detectable by TLC. Spotting was very difficult and the product(s) was not UV active as opposed to all other compounds synthesized. Similar results are reported for 8-hydroxy-2-arylquinolines[109].

> "Most of the 8-quinolinols prepared showed little or no fluorescence under ultraviolet light, while the corresponding 8-methoxy compounds generally had a strong blue-white fluorescence."

Due to the difficulties experienced at the time it was decided to protect the amine prior to demethylation. This was easily achieved by treatment of **80** with trifluoroacetic anhydride in pyridine to afford **84** in near quantitative yield. Demethylation to the trifluorohydroxy acid **85** was achieved in 57% yield by brief treatment with ten equivalents of pyridine hydrochloride at 160-70 °C[110]. Removal of the trifluoroacetamide was virtually instantaneous merely upon stirring in dilute sodium hydroxide. The result, however, was the familiar dark green (almost black) alkaline solution. Acidification with hydrochloric acid afforded a red purple solution from which a fine powder would eventually precipitated. Collection of this powder, ranging in color form red to purple to black, was very difficult as it displayed a remarkable tendency to adhere to glassware and surfaces of spatulas and funnels, etc. Proton NMR on this crude material showed the desired four aromatic doublets along with numerous extraneous resonances.



84



85

Because these results were equally disappointing still other methods of demethylation were examined. Treatment of **80** with 4.5 equivalents of potassium iodide and 9 equivalents 95% phosphoric acid at reflux[111] proved fruitless as this led only to partial saponification of the ester. These reagents had been successful in the synthesis of xanthurenic acid from its corresponding methyl ether ester[112].

Difficulty in isolation of other hydroxy and aminoquinoline carboxylic acids is well documented. 5-Aminoquinoline-6-carboxylic acid[113] was isolated from an aqueous alkaline solution by neutralization with dilute acetic acid. At first "a brown flocculent precipitate" formed followed by the amino acid as "orange or reddish brown needles". This was purified by dissolution in "caustic alkali" and precipitation with acetic acid. Application of these sequences to isolate **52** showed no improvement in purity nor in ease of formation. 5-Hydroxyquinoline-6-carboxylic acid[114] is "a dark greenish granular precipitate which dissolved in alkalies to a dark green solution and in acids to a reddish green one". This was purified by decolorizing its alkaline solution with "bone black" and acidification of the filtrate. Application of this treatment on **52** was ineffective as precipitation could not be induced.

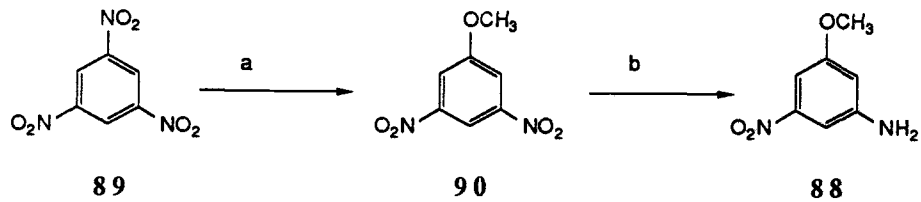
Finally, isolation by purification of the copper salt and conversion to its sodium salt was tried. This sequence has been used in the synthesis of quinaldinic acid[115] and 8-aminoquinaldinic acid[116]. An aqueous solution of crude **52** at pH 6 was treated with a slight excess of saturated copper acetate solution. Upon refrigeration a small amount of a blue green copper salt

formed. This crude paste was boiled in a slight excess of 2N sodium hydroxide and the by-product copper oxide removed by hot filtration. Upon cooling, no sodium salt of **52** precipitated. Acidification with dilute acetic acid was again ineffective.

Although all the aforementioned observations provided circumstantial evidence for the formation of **52**, no further attempts at characterization were carried out. A feeding of crude deuterium labelled material was nevertheless performed. See the Feeding Experiments section for details.

7-Amino-5-hydroxyquinaldinic Acid **53**

Entrance into the 5-hydroxyquinolines appeared very difficult as judged by their scarcity in literature. Thus it was unfortunate that the only plausible method entailed production of a mixture of wanted and unwanted isomers early in its conception. It was envisioned that cyclization of the fumarate derivative **86** would afford a mixture of quinolones **87a** and **87b** of which only **87a** could be utilized (Scheme 16). Formation of the fumarate adduct **86** required 3-amino-5-nitroanisole, **88**, a known compound[117,118]. Its synthesis was modified slightly to avoid the use of sodium sulfide. Thus **88** was produced in two steps from 1,3,5-trinitrobenzene, **89**, as shown in Scheme 17. A methanolic slurry of 1,3,5-trinitrobenzene was treated with an aqueous methanolic slurry of potassium bicarbonate[117] to afford 3,5-dinitroanisole, **90**, in 82% yield. A rather interesting mechanism has been proposed for this transformation and is shown in Scheme 18. This compound was then reduced with the triethylamine/formic acid methodology[103] previously used in the synthesis of **69a** to afford **88** in 52% overall yield for the two steps.



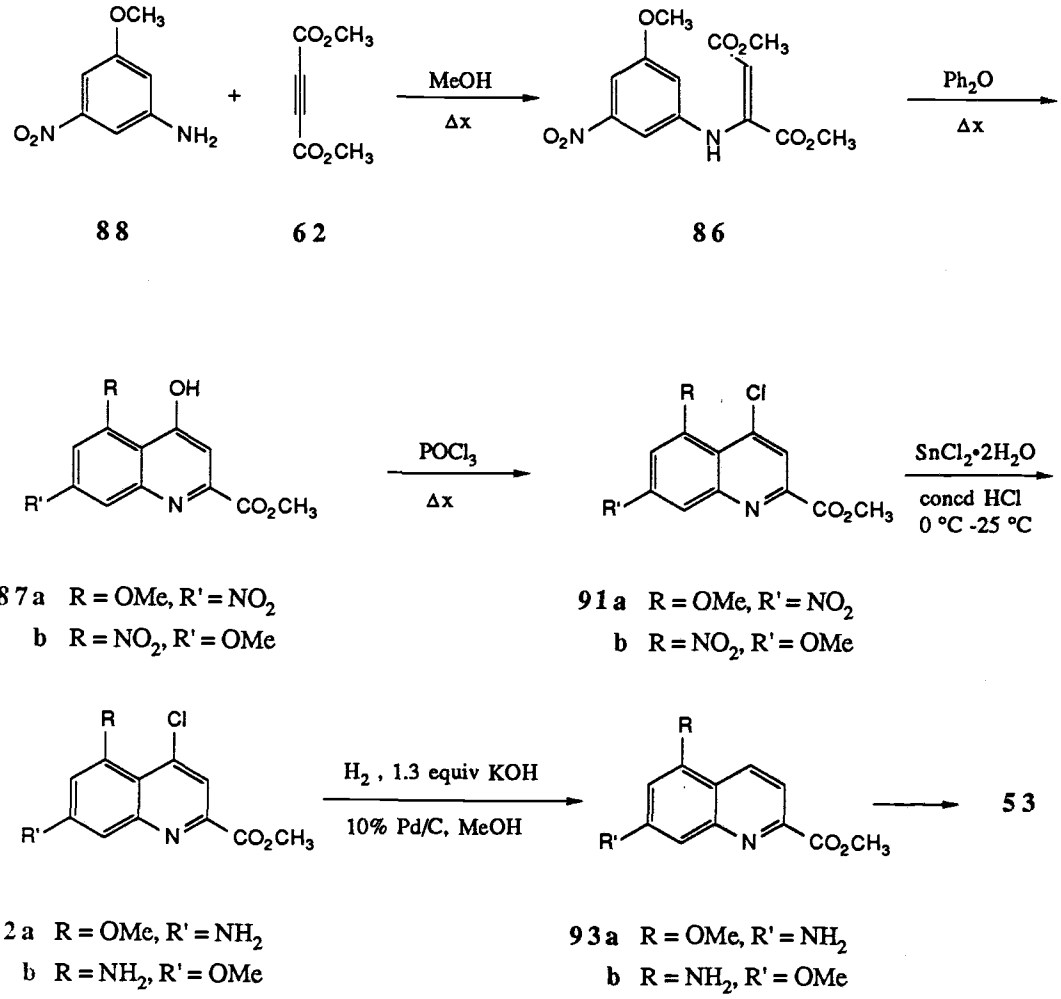
89 **90** **88**

a) 2.7 equiv $KHCO_3$, MeOH, H_2O
b) 3.3 equiv Et_3N, HCO_2H, 10% Pd/C, CH_3CN

Scheme 17. Synthesis of 3-Amino-5-nitroanisole **88**

Condensation of **88** with **62** proceeded somewhat slower than for the desmethoxy and 8-methoxy analogs but still afforded dimethyl (3'-methoxy-5'-nitroanilino) fumarate, **86**, in 86% yield. Brief treatment of **86** in diphenyl ether[74] at reflux afforded a mixture of two isomeric quinolones in roughly 2 : 1 ratio. Unfortunately, the compound believed to be the desired 5-methoxy-7-nitro isomer because of its higher R_f (0.37 in EtOAc) was the

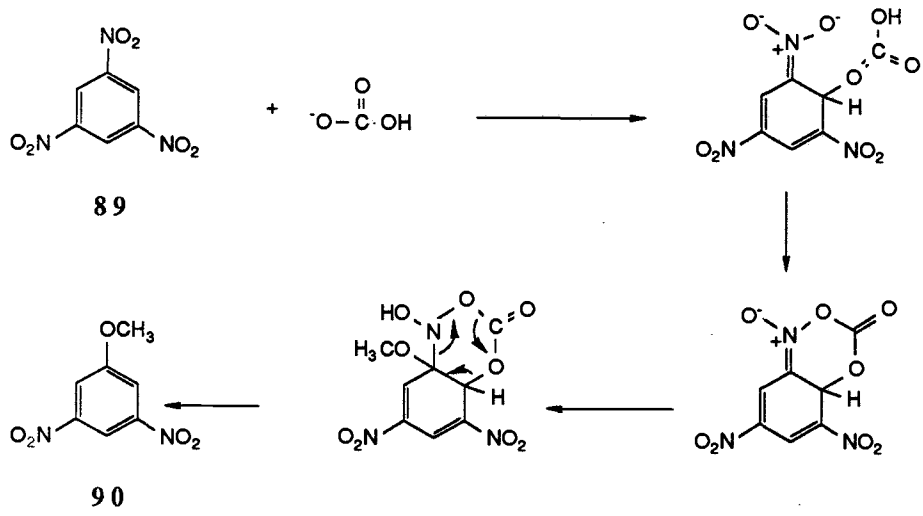
minor product. Performing the same cyclization in *ortho*-dichlorobenzene[80] led to an even greater preponderance of the lower R_f, (0.28) believed to be the 7-methoxy-5-nitro isomer. This observation parallels the earlier finding between solvent reflux temperature and the 7-nitro/5-nitro product ratio. Due to the relative insolubility of these compounds separation was not even attempted at this stage.



Scheme 16. Synthesis of 7-Amino-5-hydroxyquinaldinic Acid **53**

The isomeric mixture **87ab** was treated with phosphorus oxychloride in the usual fashion[77] to afford the chlorinated mixture **91ab**. With some difficulty, these compounds were separated by flash chromatography to afford the desired methyl-4-chloro-5-methoxy-7-nitroquinoline-2-

carboxylate, **91a**, in 27% yield and the undesired methyl-4-chloro-7-methoxy-5-nitroquinoline-2-carboxylate, **91b**, in 63% yield. Each of these compounds was then independently reduced to the chloro amine adducts **92a** and **92b** in 86% and 77% yield respectively by treatment with stannous chloride[87] as before. However in this series purification of these compounds was somewhat lengthy. It was essential to remove any trace of tin salts and/or decompose any organotin complexes prior to the reductive cleavage step. The ethyl acetate layers of **92** were washed twice with dilute sodium hydroxide to remove the majority of tin salts. The crude products were then purified by flash chromatography (EtOAc). Catalytic reduction[89] of each in alkaline methanol then afforded the respective methyl aminomethoxyquinaldinates **93a** and **93b** in 90% and 100% yield.



Scheme 18. Mechanism of Formation of **90**

To confirm the assigned regiochemistry two dimensional NMR studies of **93a** and **93b** were undertaken. The long range ^{1}H-^{13}C HETCOSY spectra of each are shown in Figures 19 and 20. Compound **93a** showed the expected three bond coupling of C-5 to the methyl ether protons and to H-4. The isomeric **93b** only had long range coupling of C-5 to H-4. The methyl ether

protons were clearly coupled to C-7. Other couplings could be seen as well and are shown in Table 8.

The synthesis of **53** was completed by treatment of **93a** with 48% HBr at reflux[119]. In this case the product crystallized out of the reaction mixture as golden needles upon cooling. The yield was somewhat erratic, ranging from 30 to 65%. The deuterated analog **53a** was synthesized as had the other labelled analogs using deuterium gas and methanol-d_1 as solvent in the reduction cleavage.

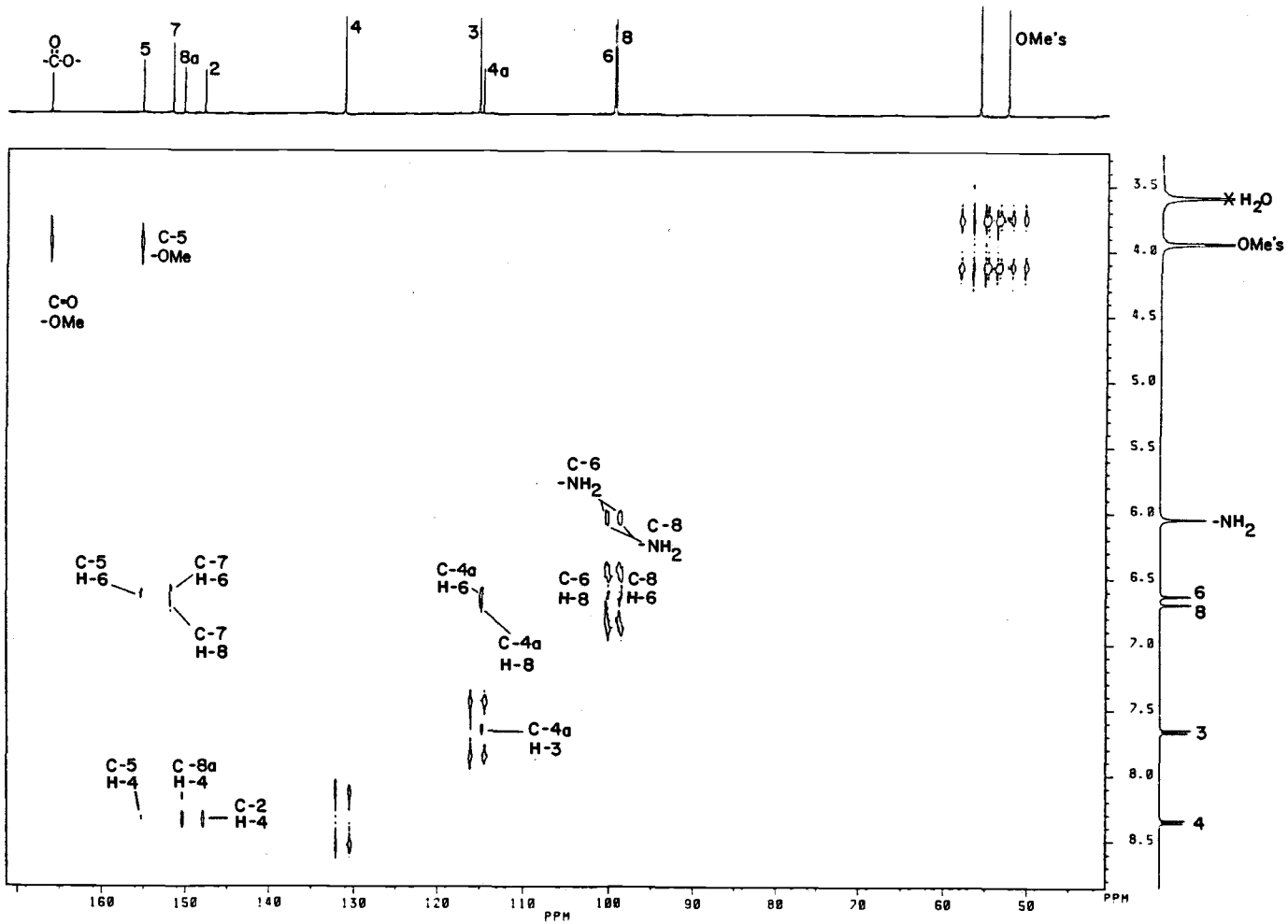


Figure 19. [^{13}C-^{1}H] LR HETCOSY NMR Spectrum of **93a**

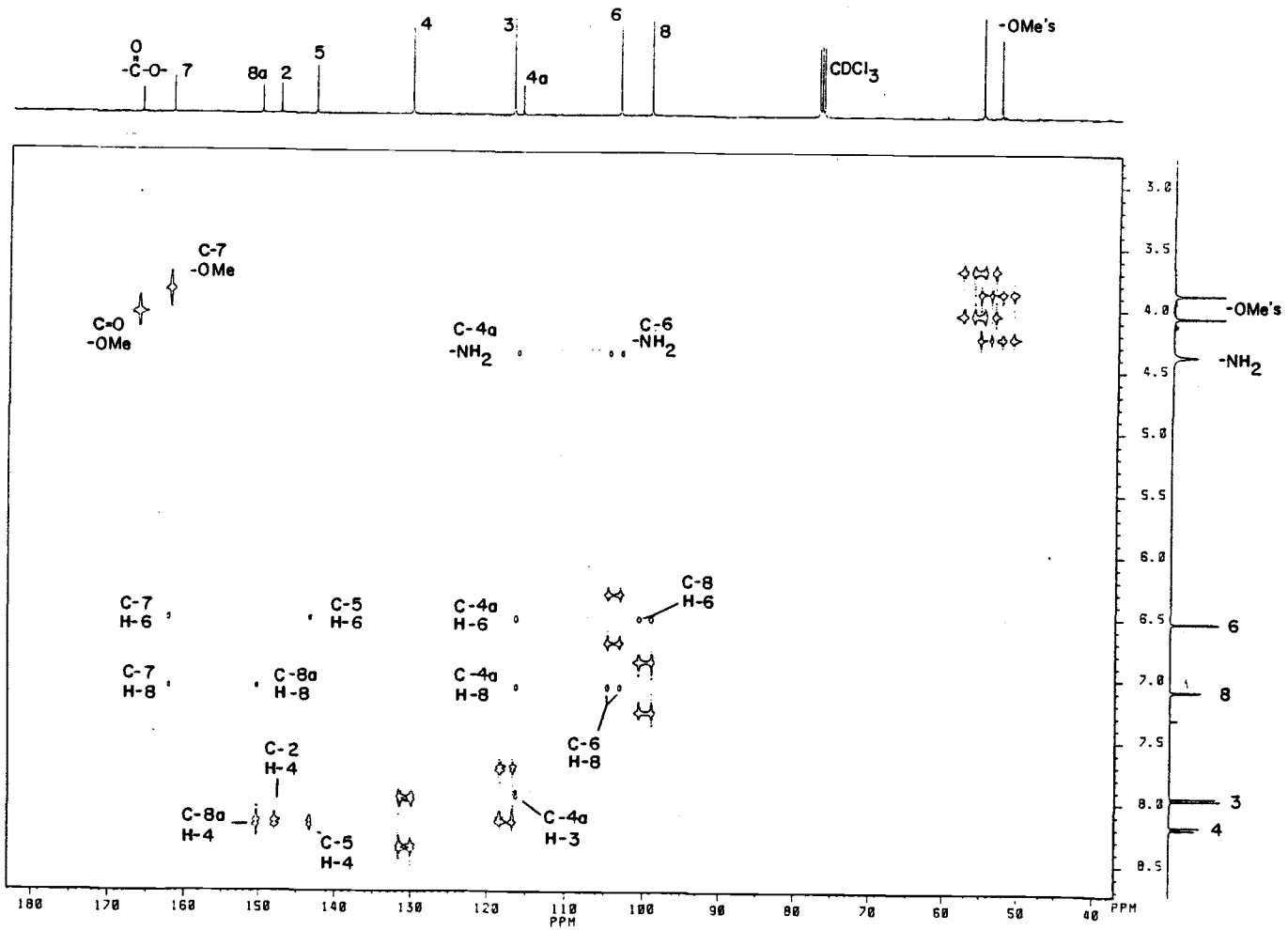


Figure 20. [^{13}C-^{1}H] LR HETCOSY NMR Spectrum of **93b**

Methyl 7-amino-5-methoxyquinoline-2-carboxylate **93a**

CARBON	HYDROGEN
carbonyl	methyl ester protons
C-5	methyl ether protons, H-4, 2 bond to H-6
C-7	2 bond to H-6 and H-8
C-2	H-4
C-8a	H-4
C-4	direct to H-4
C-3	direct to H-3
C-4a	H-3, H-6, H-8
C-6	H-8, $-NH_2$, direct to H-6
C-8	H-6, $-NH_2$, direct to H-8

Methyl 5-amino-7-methoxyquinoline-2-carboxylate **93b**

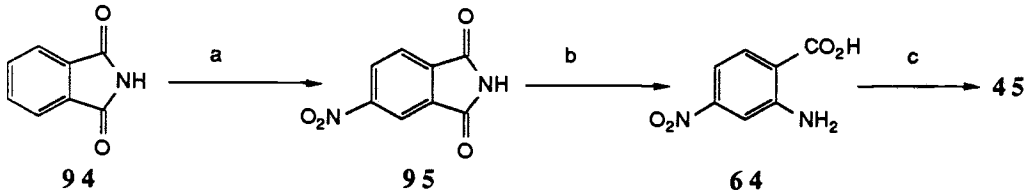
CARBON	HYDROGEN
carbonyl	methyl ester protons
C-7	methyl ether protons, 2 bond to H-6 and H-8
C-8a	H-4, 2 bond to H-8
C-2	H-4
C-5	H-4, 2 bond to H-6
C-4	direct to H-4
C-3	direct to H-3
C-4a	H-3, H-6, H-8, $-NH_2$
C-6	H-8, $-NH_2$, direct to H-6
C-8	H-6, direct to H-8

Table 8. Long Range ^{1}H-^{13}C Couplings of **93a** and **93b**

2,4-Diaminobenzoic Acid **45**

There are several syntheses of **45** reported in the literature[120,121]. However our specific needs required a synthesis which was readily amenable to introduction of a suitable label. A carbon-13 label in the benzene ring was obviously not worth considering since this would be cost prohibitive and most likely require a prearomatic synthetic intermediate. A carbon label in the carboxylic acid moiety would have proved fruitless as decarboxylation took place in our biosynthetic proposal. Therefore the use of a nitrogen label in one of the amine functionalities seemed ideal. Along these lines a known simple three step synthesis as outlined in Scheme 19 was followed. Nitration of phthalimide, **94**, with 2 equivalents of 50% (w/w) aqueous nitric acid in fuming sulfuric acid[122] afforded 4-nitrophthalimide, **95**, in 36% yield. Subsequent Hoffmann rearrangement[123,124] of **95** by treatment with aqueous alkaline sodium hypochlorite afforded 4-nitroanthranilic acid, **64**, in 54% after recyrstallization from 95% ethanol. Catalytic reduction in methanolic HCl followed by precipitation from ethyl ether afforded **45** as its dihydrochloride salt in 92% yield. This salt was not very stable and turned from whitish gray to purplish brown and finally to black over a 5 or 6 month period. Substitution of dichloromethane for ethyl ether to affect precipitation afforded **45** as white crystals of high purity.

The labelled analog [4-^{15}N] 2,4-diaminobenzoic acid, **45b**, was synthesized in similar fashion using 99% enriched ^{15}N nitric acid in the nitration step. However in this case the crude labelled 4-nitroanthranilic acid was not recrystallized but instead taken on to the reduction step. The overall yield was 22%.



94 **95** **64**

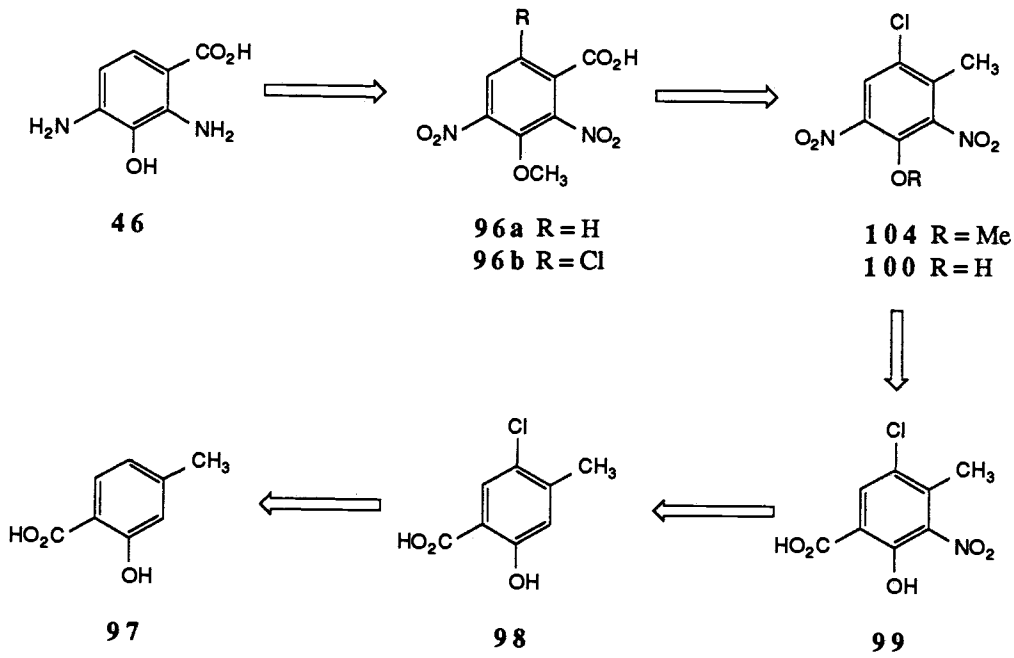
a) 2.2 equiv 50% (w/w) aqHNO$_3$, fuming H$_2$SO$_4$, 0 °-25 °C
b) 5.25% NaOCl, NaOH
c) H$_2$ gas, 10% Pd/C, MeOH, concd HCl

Scheme 19. Synthesis of 2,4-Diaminobenzoic Acid **45**

2,4-Diamino-3-hydroxybenzoic Acid **46**

Synthesis of **46** represented an enormous synthetic challenge for, among other reasons, introduction of a suitable label. An oxygen label at the 3 position would have been inferior for two reasons. Firstly, if positive incorporation of ca 1-2% into STN were anticipated its detection would be virtually impossible. The expected upfield ^{13}C-^{18}O shifted resonance would be too small to be observed above background by ^{13}C spectroscopy. Secondly, in all likelihood any label incorporated would be lost from the C-8 position via exchange as discussed in the oxygen fermentation section. As was the case with **45**, introduction of a nitrogen label at the 4 position was the only feasible possibility.

It seemed logical that **46** could be easily obtained from reduction and deprotection of the dinitro adduct **96a** or **96b** (Scheme 20). The methyl ester of **96a** is reported in Russian literature[125] but is ultimately derived from 2,3,4-trinitrotoluene. This was totally unacceptable for our needs as introduction of label would have been impossible.



46 **96a** R=H, **96b** R=Cl, **104** R=Me, **100** R=H, **97**, **98**, **99**

Scheme 20. Retrosynthetic Analysis of **46**

Approach 1

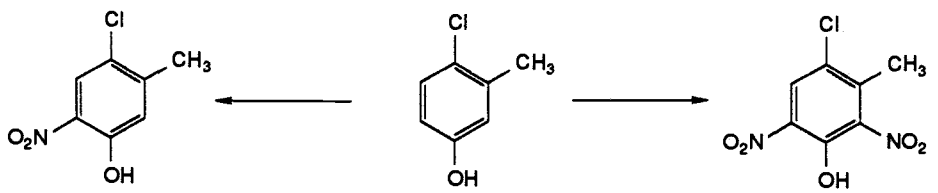
Carrying the retrosynthesis further, sequential introduction of nitro groups was desirable. Along these lines, employment of established electrophilic aromatic substitution chemistry seemed appropriate. A sequence involving an ipso substitution (similar to that tried in the synthesis of 4-chloro-2,6-dinitrophenol **69b**) was devised. These compounds are reported in the literature.

Attempted chlorination of the commercially available 4-methylsalicylic acid, **97**, with sulfuryl chloride (SO_2Cl_2)[126] in methylene chloride or carbon tetrachloride gave a mixture of compounds. Conversion to *p*-chloro-*m*-cresotinic acid, **98**, was eventually cleanly achieved in 57% yield simply by bubbling chlorine gas into a rapidly stirred acetic acid solution of **97**[127].

Attempted mononitration of **98** in a nitric acid/acetic acid mixture at room temperature gave no reaction. Treatment of **98** with concd nitric acid alone led to consumption of starting material but formed several products. Among these were probably **99** and **100**. Use of concd sulfuric acid and fuming sulfuric acid did not lead to any appreciable formation of isolable products. Because the stepwise control of mononitration/dinitration of **99** proved impossible this route was abandoned.

Approach 2

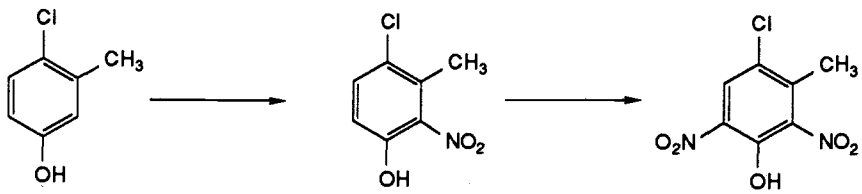
A new route (Scheme 21) from *p*-chloro-*m*-cresol, **101**, was adopted based on its easy conversion to either 6-chloro-4-nitro-*m*-cresol, **102**, or 6-chloro-2,4-dinitro-*m*-cresol, **100**, depending on conditions[128].



102 **101** **100**

Scheme 21. Synthesis of **100** and **102** From *p*-Chloro-*m*-cresol **101**

Thus, when a solution of **101** in 80% acetic acid was treated with 1.4 eqiuv of 50% (w/w) aq nitric acid at room temperature for ten minutes **102** was formed in 39% yield. When a solution of **101** in glacial acetic acid was treated with 2.2 equiv of concd nitric acid at 75 °C for three hours **100** was formed in 81% yield. Interestingly, however, when the mononitro adduct **102** was subjected to the identical conditions, no dinitro adduct **100** could be detected. This can be plausibly explained by alteration of the order of nitration in conversion of **101** to **100** under these conditions; that is **101** may first be nitrated to **103** and then further nitrated to **100** as shown in Scheme 22.



101 **103** **100**

Scheme 22. Hypothetical Order of Nitration From **101** to **100**

Trace amounts of **100** were eventually obtained from **102** by treatment with fuming nitric acid at 65 °C. Because of this low yield and harsh conditions it was felt that the one step dinitration was best even though this would require twice as much ^{15}N labelled nitric acid for the labelled synthetic sequence.

The next step required was oxidation of the benzylic methyl group to a carboxylic acid group. The presence of the phenol moiety, however, required that it be protected to avoid possible overoxidation to a quinone species. A benzyl group appeared reasonable as it is stable to a variety of oxidants[129] including CrO_3 at pH 1, CrO_3 in pyridine, and $Tl(NO_3)_3$. Furthermore it can easily be removed concurrent with reduction of the nitro

and chlorine functionalities. Surprisingly benzylation of **100** could not be achieved under the variety of conditions summarized below.

BnCl, NaOH, $CHCl_3$, MeOH	no rxn.
BnBr, NaH, benzene, room temp	no rxn.
BnBr, NaH, DMF, room temp	no rxn.
BnBr, Ag_2O, $CHCl_3$, Δx	no rxn.

This alkylation problem proved to be steric in nature as **100** was easily methylated to **104** in 79% by treatment with methyl iodide and silver oxide[90d,91] as described previously.

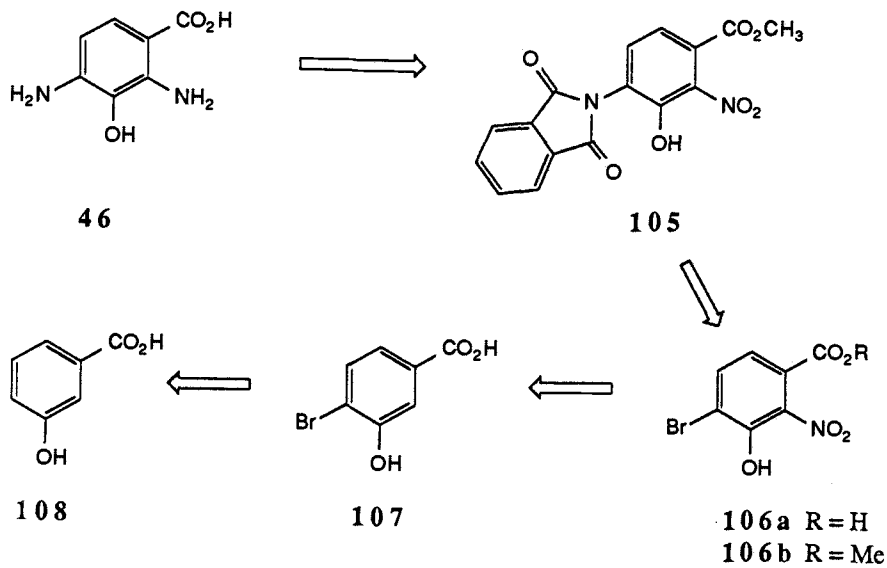
Benyzlic oxidation of **104** to 6-chloro-2,4-dinitro-3-methoxybenzoic acid, **96b**, failed miserably under a plethora of reaction conditions. Mild oxidation with mercuric nitrate in nitric acid at reflux[130] led to varying results depending on the amount of oxidant used. These permutations are listed below.

Amount of $Hg(NO_3)_3$	Rxn. Time	Result
0.1 equiv	120 h	no rxn.
0.2 equiv	36 h	one product and s.m.
1.0 equiv	20 h	apparent decomposition
none	24 h	no rxn.

Treatment of **104** with potassium dichromate in sulfuric acid at reflux[131] for thirty minutes gave no product. Oxidation with alkaline aqueous permanganate[132] afforded two products as judged by TLC (R_f 0.15 and 0.35 in EtOAc). A small amount of the higher R_f compound was isolated by flash chromatography. Infrared analysis (KBr) showed peaks at 1605, 1540, 1350, abd 1240 cm^{-1}. The expected carboxylate resonance was clearly absent and closer scrutiny revealed this product to be **100**, the result of addition-elimination at the highly reactive position *ortho* to both nitro groups. This compound cospotted with the product from the earlier oxidation attempts with mercuric nitrate. Identical results were also obtained using permanganate in a 1:1 mixture of pyridine/water[133]. Believing this oxidation to be futile this approach was also abandoned.

Approach 3

An alternative novel approach toward **46** using potassium phthalimide instead of nitric acid as the ^{15}N source was devised. The retrosynthetic scheme is shown in Scheme 23. Here it was hoped that the phthalimido adduct **105** could be formed by nucleophilic displacement of bromide from **106b**. Such Ullmann couplings have precedent on simple haloarenes[134].



Scheme 23. Retrosynthetic Analysis of **46** From Ullmann Coupling

4-Bromo-3-hydroxybenzoic acid, **107**, was synthesized in ca 20% yield by reaction of commercially available 3-hydroxybenzoic acid, **108**, with one equivalent of bromine in acetic acid[135]. Although the yield was poor this reaction was still very cost effective and easily carried out on massive scale. The bromide **107** was then converted to the ester **106b** by a two step literature procedure[136]. Nitration of **107** proceeded smoothly to afford a mixture of **106a** and trace amounts of another nitro isomer. Treatment of the crude reaction with methanolic HCl then afforded pure **106b** in 45% yield after recrystallization from methanol/water.

Having the bromohydroxynitro ester **106b** at hand, trials on Ullmann

coupling were undertaken. Unfortunately, none of the combinations tried afforded tractable products. These unsuccessful conditions are listed below.

1.2 equiv Phth, 2.0 equiv CuI in DMA, 165 °C, 22 h, N_2 atm	many products
1.2 equiv Phth, no catalyst in $CHCl_3$, Δx, 23 h, N_2 atm	no rxn.
1.2 equiv Phth, 1.0 equiv CuI in $CHCl_3$, Δx, 3 h, N_2 atm	no rxn.
2.0 equiv Phth, no catalyst in DMA, 165 °C, 45 h, N_2 atm	inconclusive
1.2 equiv Phth, 1.2 equiv Cu_2O in *s*-collidine, Δx, 2 h, N_2 atm	decomposition

Due to the many difficulties encountered the attempted synthesis of **46** was abandoned. However, as will be discussed in the Biosynthetic Studies section, it is highly unlikely that **46** is a viable intermediate in the biosynthesis of **1**.

Biosynthetic Studies

Stable Isotope Feeding Experiments

Mixed results were obtained from feeding experiments of **45a**, **51a**, **52a**, and **53a** conducted with *S. flocculus* fermentations. All of these putative precursors were pulse fed over varying schedules which are detailed for each individual experiment in their respective sections and/or in the Experimental Section.

[4-2H] 7-Aminoquinaldinic Acid **51a**

Trial 1 Initially, a total of 123.3 mg (652.3 μmol) of the labelled amino acid **51a** was administered as its sodium salt in three pulses at 36 h, 48 h, and 60 h after inoculation. After a total of 92 h, standard work-up of the 3 x 500 mL *S. flocculus* fermentations afforded 22.2 mg (43.8 μmol) of streptonigrin, **1b**, as determined by UV. Analysis of a 14 mg (27.6 μmol) sample by ^{2}H NMR revealed a broad singlet at ca. δ8.1 ppm relative to solvent DMSO (δ2.49). Incorporation was determined to be 0.2% by application of the following formulae:

400 μL DMSO in NMR tube = 5.63 μmol of DMSO in NMR tube

$$5.63\ \mu\text{mol of DMSO} \times \frac{6\ \text{mmol hydrogen}}{1\ \text{mmol DMSO}} \times 0.016\% = 5.27\ \mu\text{mol of } ^2\text{H in solvent}$$

Integration of the peak area at δ8.1 gave a value of 16% that of the DMSO peak.

5.27 μmol of ^{2}H in solvent x 16% = 0.8 μmol deuterium in STN sample **1b**

$$\%\ \text{enrichment} = \frac{\text{Amount of deuterium in sample}}{\text{Amount of STN in tube}} \times 100\%$$

$$= 0.8\ \mu\text{mol}/27.6\ \mu\text{mol} \times 100\% = 2.9\%$$

$$\text{dilution} = 100/\text{enrichment} = 100/2.9 = 34.5$$

$$\%\ \text{incorporation} = \frac{\mu\text{mol of STN produced}}{\mu\text{mol substrate fed} \times \text{dilution}} \times 100\%$$

= 43.8 μmol/(652.4 μmol x 34.5) x 100% = 0.2%

Trial 2 Due to the low level of incorporation in the first trial the feeding was repeated. In this case a total of 135.2 mg (715.3 μmol) of **51a** was administered as its sodium salt over the same schedule described in Trial 1. Standard work-up of these fermentations afforded 13.2 mg (26.0 μmol) of **1b**. Interestingly, upon analysis of an 8.5 mg sample, no incorporation was detected this time.

Trial 3 A third and decisive feeding experiment was performed. In this case an additional pulse was added at 28 h and the last pulse administered at 58 h instead of 60 h. A total of 172.1 mg (910.8 μmol) of **51a** was administered as its sodium salt. Standard work-up afforded 20.6 mg (40.6 μmol) of **1b**. This new feeding schedule appeared effective as analysis by ^{2}H NMR showed a sharp singlet at 8.23 ppm relative to solvent DMSO. Using the above formulae, incorporation was determined to be 1.4%. This spectrum is shown in Figure 21.

[4-^{2}H] 7-Amino-8-hydroxyquinaldinic Acid **52a**

The feeding schedule used in Trial 3 for **51a** was followed for this experiment. After 92 h, standard work-up of 3 x 500 mL *S. flocculus* fermentations which had been administered approximately 150 mg (731.7 μmol) of crude **52a** as its sodium salt afforded ca 22 mg (39 μmol) of streptonigrin, **1c**, as determined by UV. Analysis of a 12 mg (24 μmol) sample by ^{2}H NMR revealed that no incorporation had taken place.

[4-^{2}H] 7-Amino-5-hydroxyquinaldinic Acid **53a**

Here again the successful feeding sequence used in Trail 3 of the feeding of **51a** was followed. After 92 h, standard work-up of 3 x 500 mL *S. flocculus* fermentations which had been administered 188.8 mg (921.0 μmol) of **53a** as its sodium salt afforded 25.1 mg (49.5 μmol) of streptonigrin, **1d**, as determined by UV. Analysis of a 14.6 mg (28.8 μmol) sample by ^{2}H NMR revealed that no incorporation had taken place for this acid, either.

[4-^{15}N] 2,4-Diaminobenzoic Acid **45a**

For this experiment the same feeding sequence as described above was employed except for the first pulse. This was administered at 24 h instead of

28 h. Thereafter the timing was the same throughout the fermentation. Standard work-up of 3 x 500 mL *S. flocculus* fermentations which had been administered 122.5 mg (544.4 μmol) of **45a** as its sodium salt afforded 37.6 mg (74.2 μmol) of streptonigrin, **1e**, as determined by UV. Analysis of a 22.5 mg (44.4 μmol) sample by standard ^{15}N NMR showed a sharp singlet corresponding to the C-7 amine at 73.6 ppm relative to external enriched [^{15}N] aniline, 56.5 ppm. The specific enrichment could not be calculated due to NOE because of proton decoupling. No resonance for the C-5' amine could be detected but, in earlier work[49a], both amine peaks were detected at equal intensity in a natural abundance spectrum of STN. The C-7 nitrogen resonance was also observed in the enriched and natural abundance samples by using a refocused decoupled INEPT sequence[137]. This spectrum is shown in Figure 22. The peak at ca δ70.1 ppm is an impurity present in the [^{15}N] aniline. The INEPT sequence gave a signal-to-noise ratio approximately three times greater than that of the standard experiment. The C-5' amine was not observed in this case either, presumably due to rapid proton exchange eliminating the possibility of efficient polarization transfer. It is of interest to note that long range coupling between C-5' and its amine protons was absent in the two dimensional ^{13}C-^{1}H LR HETCOSY NMR spectra described earlier in this work.

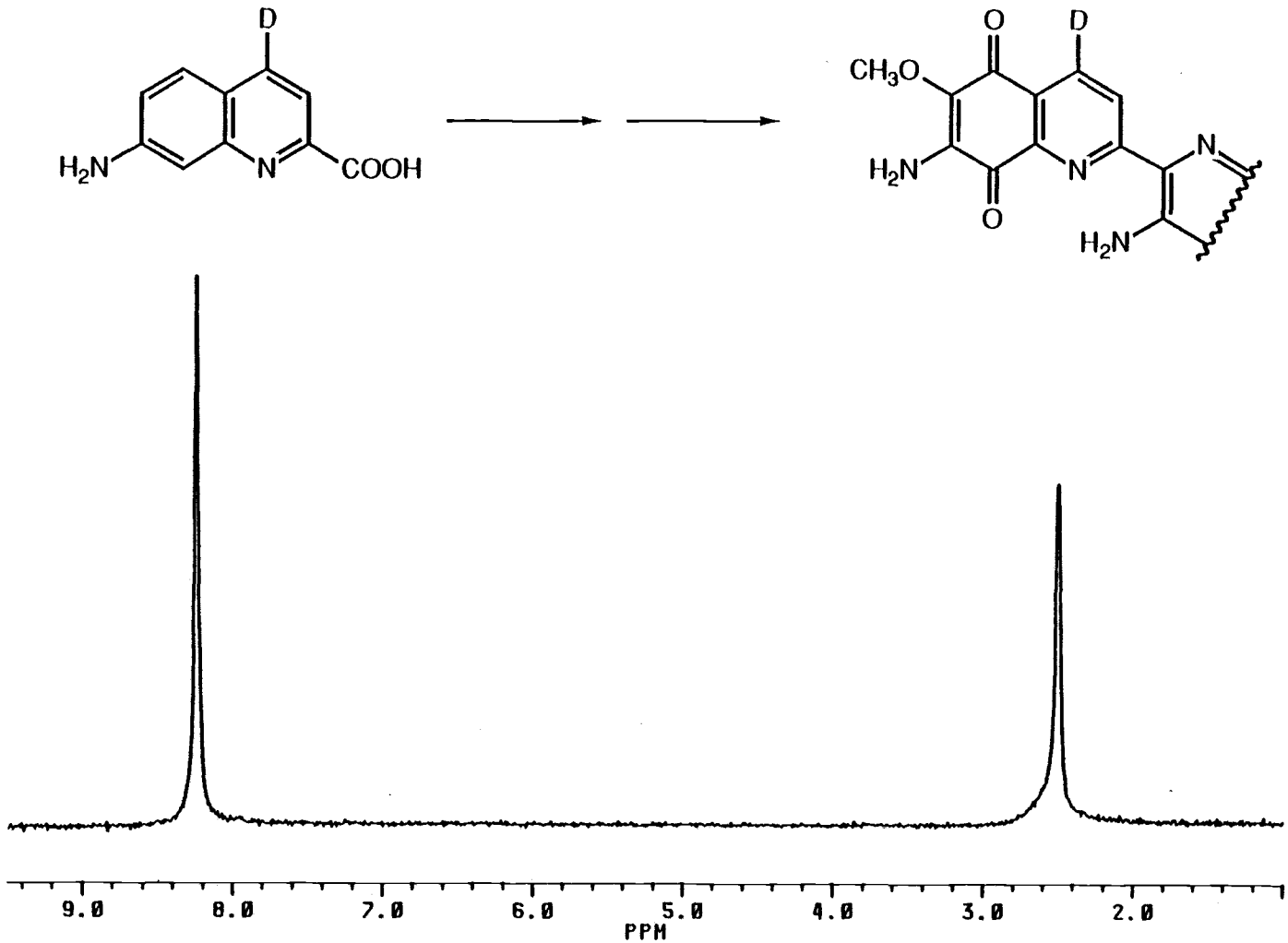


Figure 21. 61.4 MHz ^{2}H NMR Spectrum of Streptonigrin, **1b**, From The Feeding Of **51a**

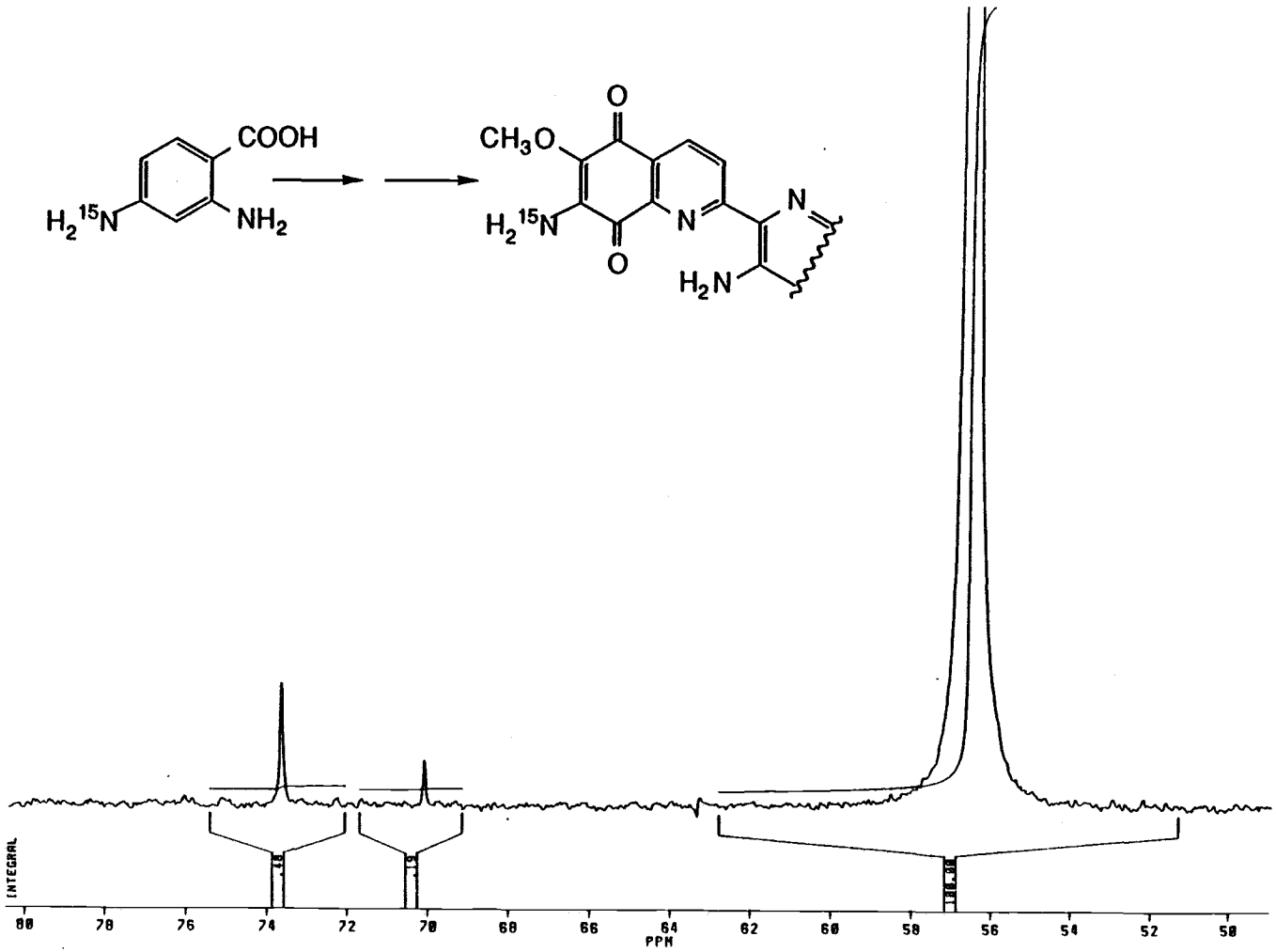


Figure 22. 40.5 MHz ^{15}N INEPTRD NMR Spectrum of Streptonigrin, **1e**, From The Feeding Of **45a**

Radioactive Isotope Trapping Experiments

It was possible that these aforementioned incorporations of **45a** and **51a** were merely the result of fortuitous incorporation; that is, the organism may have mistaken the introduced compounds for the actual intermediates which most likely would have been of similar structure. The organism then may have been able to metabolize these compounds and subsequently incorporate them into streptonigrin. To verify their incoporations as relevant, trapping experiments with these compounds were undertaken. [1-^{14}C] Erythrose[138] (specific activity of 8 mCi/mmol) was used as the radioactive precursor in both cases.

7-Aminoquinaldinic Acid Trap

Three 50 mL fermentations of *S. flocculus* were terminated at 2 h, 4 h, and 6 h after the administration of [1-^{14}C] erythrose, which was performed 12 h after inoculation. In each case a quantity of authentic **51** was added at the time of termination, the cells were broken by sonication, and **51** reisolated from the resulting mixture as described in the Experimental Section. For the 2 h trap, **51** was isolated as its acetamido methyl ester. For the 4 h and 6 h traps, **51** was isolated directly without derivatization.

With regard to the 2 h trap, after six recrystallizations of the acetamido methyl ester only 0.0658% of the total activity fed remained. A constant molar specific activity was never attained and clearly this sample was not significantly radioactive. The 4 h trap was equally cold; after just two recystallizations of **51** only 0.059% of the total activity remained having a specific activity of just 7,286 dpm/mmol. The 6 h trap work-up was likewise disappointing, having retained only 0.082% of the total activity after three recrystallizations.

There are several plausible explanations for the lack of radioactivity in these samples. Firstly, it is quite possible that the timing of the trap was incorrect. Endogenous biosynthesis of **51** may not have been occurring at the time of the work-up of the fermentations. Therefore it could not have been recovered from the broth in radioactive form. It is also possible that **51** was not a true intermediate in the biosynthesis of streptonigrin. This possiblity will be discussed in greater detail in the Analysis Section.

2,4 Diaminobenzoic Acid Trap

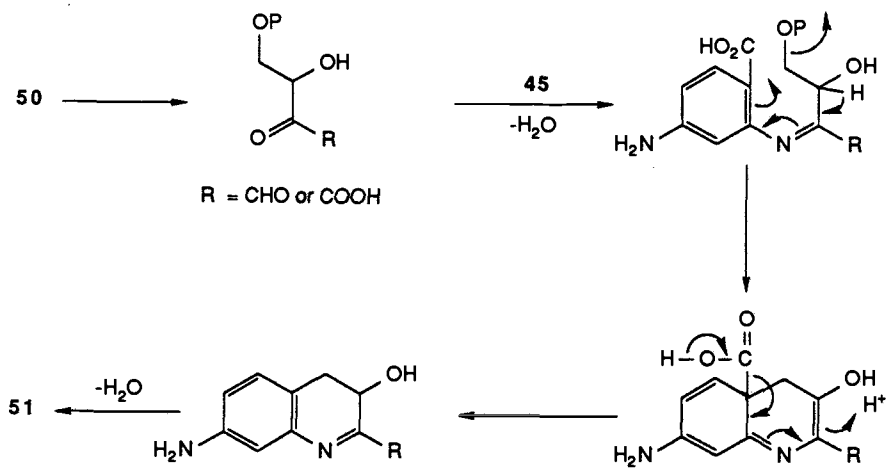
Here again three 50 mL fermentations of *S. flocculus* were terminated at 2 h, 4 h, and 6 h after the administration of [1-^{14}C] erythrose, **50a**, which was performed 12 h after inoculation. In each case a quantity of authentic **45** was added at the time of termination and reisolated from the fermentation medium as its bisacetamide as described in the Experimental Section. Fortunately the 2 h trap was radioactive having retained 0.2% of the total activity with a constant specific molar radioactivity of 23,000 dpm/mmol. This result, coupled with the intact incorporation of **45a** indicated that **45** was a true intermediate in the biosynthetic pathway toward streptonigrin.

Analysis of The Feeding and Trapping Experiments

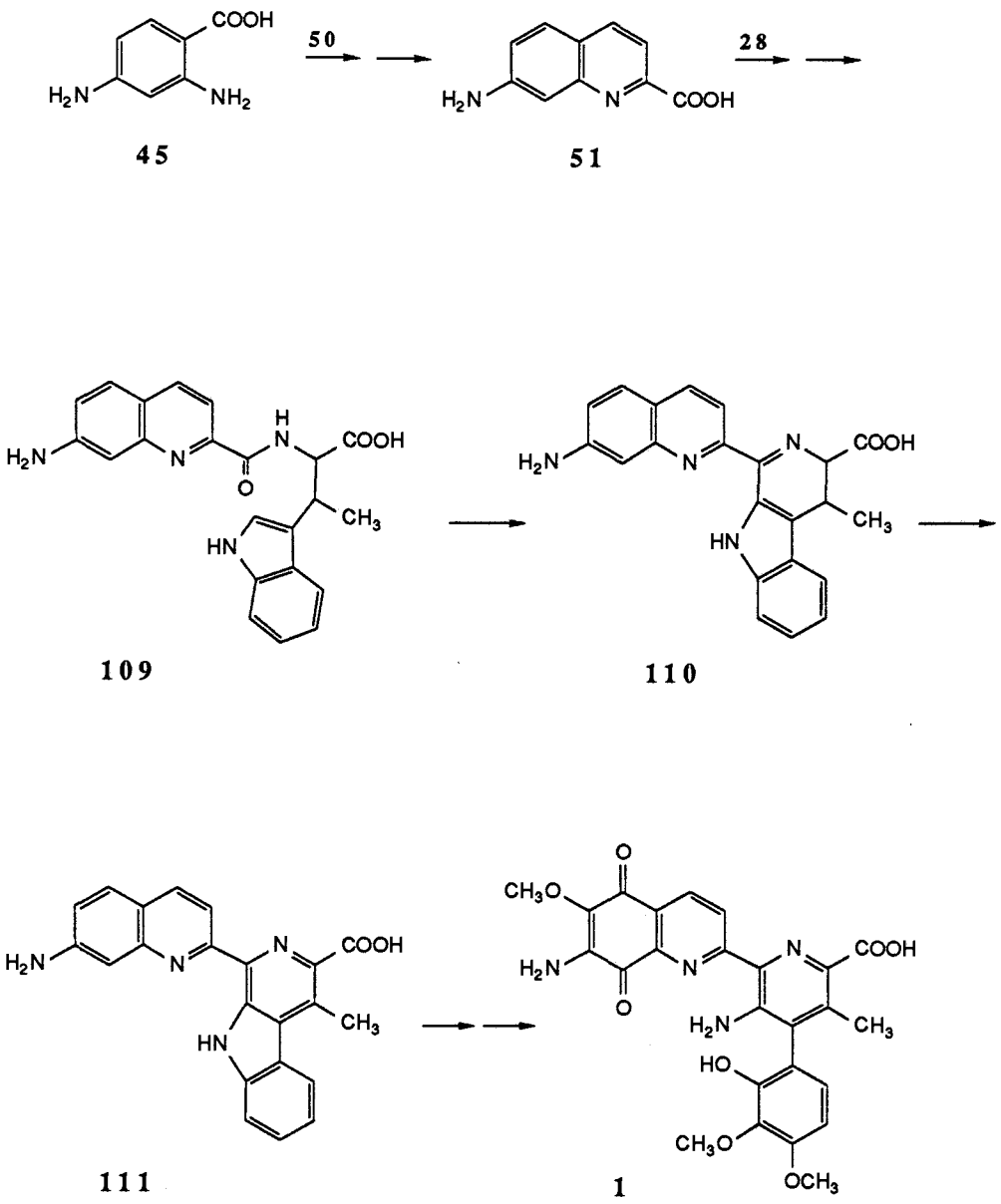
The positive incorporation of **45a**[139] and the successful radioactive trapping of its bisacetamide prove the intermediacy of the novel aromatic amino acid **45**. The incorporation **51a**[139] but apparently not **52a** and **53a** suggested that the biosynthesis of streptonigrin followed the course originally depicted in Pathway A of Scheme 4. That is 2,4-diaminobenzoic acid, **45**, is first coupled with erythrose-4-phosphate or derivative thereof (R = COOH) and subsequently cyclized to form 7-aminoquinaldinic acid, **51** as depicted in Scheme 24. This mechanistic pathway may be viewed as analogous to tryptophan biosynthesis[140]. Thereafter **51** may couple with β-methyl tryptophan, **28**, forming the amide **109** as shown in Scheme 25. This could then cyclize in a Bischler-Napieralski fashion to afford the dihydopyridine compound **110**. Dehydrogenation would then afford the fully aromatic compound **111**. Although no rigorous support exists it seems reasonable that the A and D-ring oxidations would occur at this stage. Subsequent β-carboline cleavage and methylations would afford streptonigrin, **1**. Unless a metabolic grid exists, the incorporation of **51** into streptonigrin effectively eliminates the hydroxy amino acids **46** and **49** as possible intermediates.

The possibility remains, however, that incorporation of **51** into streptonigrin may have been a fortuitous event. It may be that this acid was

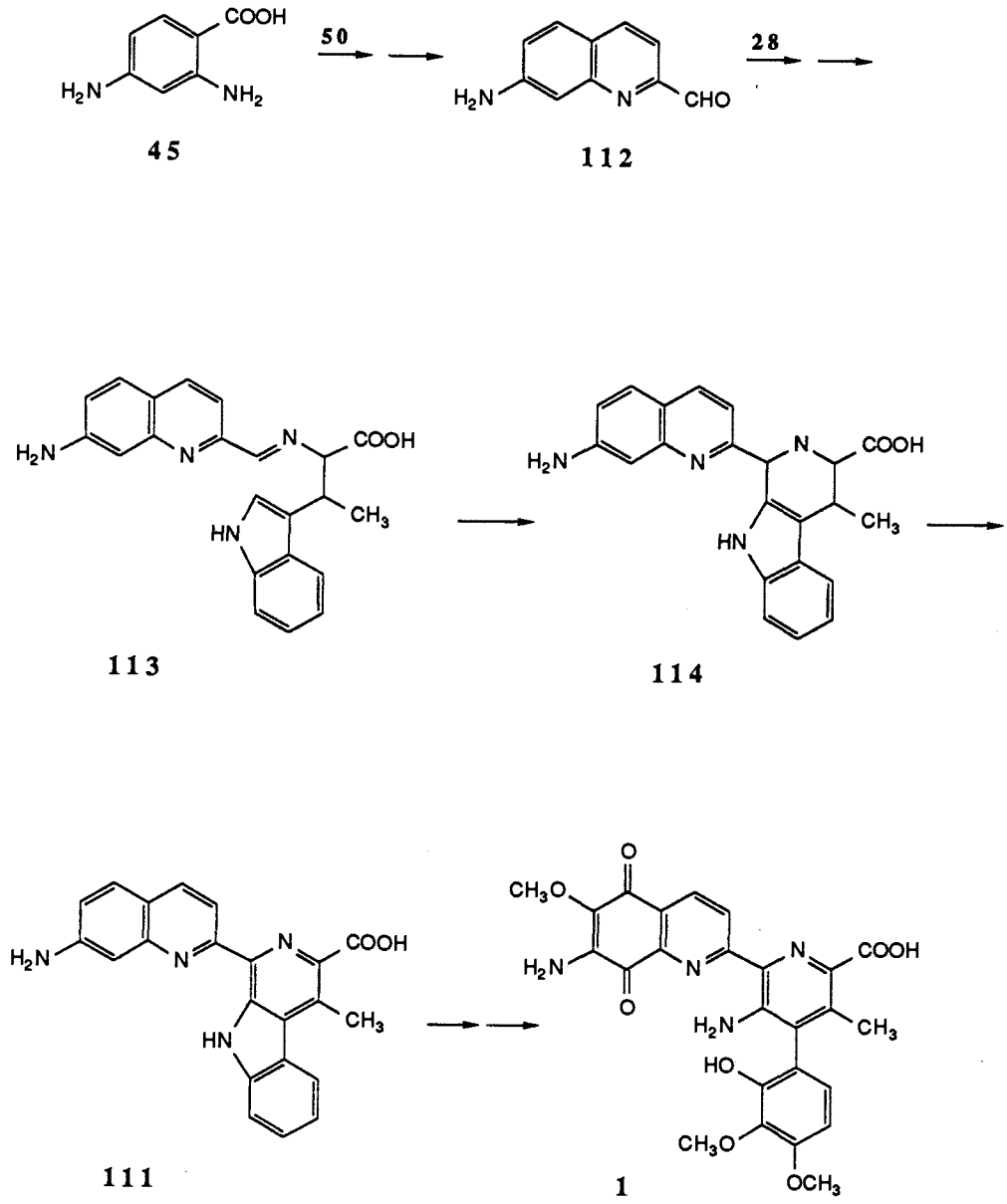
first converted to a true intermediate and subsequently incorporated. One possibility was a reduction to 7-aminoquinoline-2-carboxaldehyde **112**. This could easily be biosynthesized as shown in Scheme 24 with R = CHO. This aldehyde could then have condensed with β-methyltryptophan, **28**, to form an imine, **113** as shown in Scheme 26. Subsequent cyclization in a Pictet-Spangler fashion could afford the tetrahydropyridine **114**. Two dehydrogenations would then afford **111** and this could be further transformed as described above to afford **1**.



Scheme 24. Proposed Biosynthesis of 7-Aminoquinaldinic Acid, **51**



Scheme 25. Proposed Biosynthetic Sequence To Streptonigrin Via 7-Aminoquinaldinic Acid, **51**



Scheme 26. Proposed Biosynthetic Sequence To Streptonigrin Via 7-Aminoquinoline-2-carboxaldehyde, **112**

Miscellaneous

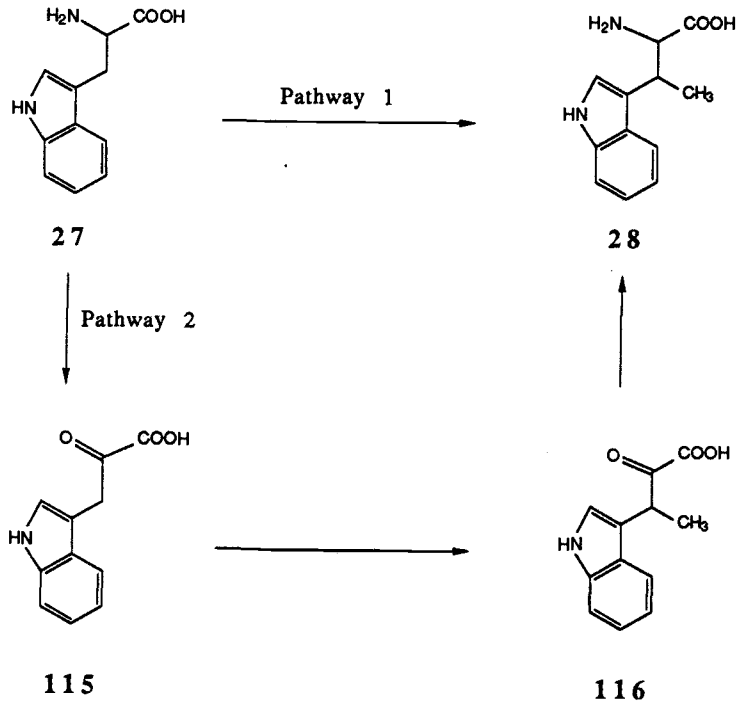
[α-^{15}N, α-^{13}C] Tryptophan 27a Feeding

During our biosynthetic investigations, we were also interested in gaining further insight into the method of methylation of tryptophan, **27**, to β-methyltryptophan, **28**, prior to incorporation into streptonigrin. Two possibilities existed; 1) direct methylation at the β-position via a pyridoxal pyrophosphate complex or 2) transamination to indole-3-pyruvic acid, **115**, and methylation at the now activated β-position (via keto-enol tautomerism) followed by transamination to **28**.

Interestingly, other workers[50] have showed that, in cell free extracts, a metabolic grid exists and that both pathways are operative (Scheme 27). Methylation of **27** by a C-methyltransferase via a pyridoxal pyrophosphate complex afforded **28**. Transamination by a tryptophan transaminase to **115** also took place followed by methylation to β-methylindole-3-pyruvic acid, **116**, and subsequent transamination to **28**.

If direct methylation were operative in the whole cell system, then the resultant carbon-nitrogen bond between N-1' and C-2' of STN should have remained intact throughout the biosynthesis. To test this, a feeding the labelled amino acid **27a** was planned. This compound had been synthesized and fed previously in these laboratories but under different conditions[45]. Previously **27a** was introduced by itself and non-specific transamination could have resulted in loss of label and accounted for the lack of detection of any ^{15}N in the resultant antibiotic. Also, the spectrum was acquired in DMSO-d_6, in which the coupling between ^{13}C and ^{15}N may have been to small to be detected. In the present feeding we planned to administer **27a** along with **115** to minimize or possibly eliminate loss of label through non-specific transamination. This methodology has been employed in studies on the biosynthesis of cytochalasins[141]. We also planned to acquire the ^{13}C NMR spectrum in TFA-d_1. It is well documented[142] that a protonated nitrogen can have a profound increase on the magnitude of the ^{13}C-^{15}N coupling constant. Examination of the ^{13}C NMR spectrum of STN isolated from this feeding showed an enrichment of 1.5% for the resonance at 128.1 ppm using the C-methyl resonance as the normalized reference. However no flanking ^{13}C-^{15}N satellites could be observed. This implied that transamination to **115** must occur prior to methylation as shown in Pathway

2. It is possible, however, that the rate constants for the two pathways of the metabolic grid were sufficiently different that only a small portion of methylation proceeded via Pathway 1. The resultant minor population of **28** with an intact ^{13}C-^{15}N bond may have been below the limits of detection by ^{13}C NMR.



Scheme 27. Possible Routes For The Conversion Of Tryptophan To β-Methyltryptophan

Experimental

General

^{1}H NMR and ^{13}C NMR were recorded either on a Bruker AM 400 (400.13 MHz and 100.6 MHz, respectively) or on a Varian FT-80A (80 MHz and 20 MHz, respectively). ^{15}N NMR and ^{2}H NMR spectra were obtained on the Bruker AM 400 at 40.5 MHz and 61.4 MHz, respectively. All chemical shifts for ^{1}H and ^{13}C spectra are reported in parts per million relative to external tetramethylsilane (Me_4Si, δ 0.00). Chemical shifts for ^{15}N spectra are relative to external ^{15}N enriched aniline (MSD Isotopes). Chemical shifts for ^{2}H spectra are relative to the natural abundance deuterium resonance of the protic solvent ($CHCl_3$ or DMSO). Infrared spectra were recorded in wavenumbers on either a Nicolet 5DXB FT-IR or a Perkin-Elmer 727B spectrometer. Low resolution mass spectra were taken on a Varian MAT CH-7 spectrometer with a System Industries 150 data system. High resolution mass spectra were taken on a Kratos MS 50 TC spectrometer. UV spectra were measured in methanol on either a Cary 210 or an IBM 9420 spectrometer. Melting points were taken on a Büchi melting point apparatus and are uncorrected. Elemental analyses were performed by R. Johnson at Desert Analytics (Tucson, AZ).

Flash chromatography was carried out on silica (EM Reagents, Keiselgel 60, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on precoated Keiselgel 60 F_{254} (either 0.2 mm aluminum sheets or 0.25 mm glass plates) and visualized by long and/or short wave UV. Preparative thin layer chromatography (PLC) was carried out on precoated Keiselgel 60 F_{254} glass plates (20 cm x 20 cm x 2mm).

Materials

Solvents for the routine acquistion of NMR spectra were purchased from either Aldrich Chemical Company, Inc. (Milwaukee, WI), Stohler/KOR Stable Isotopes (Cambridge, MA), or Cambridge Isotope Laboratories (Woburn, MA). Deuterium gas (99.5 atom % D) was purchased from Matheson (Newark, CA), $^{18}O_2$ gas (50% enriched) from Cambridge Isotope Laboratories, (^{15}N) nitric acid (50% w/w, 99% enriched), from Stohler/KOR and (^{14}C) potassium

cyanide from New England Nuclear (Boston, MA). 1,3,5-Trinitrobenzene was purchased from Dixon Fine Chemicals (Alberta, Canada).

All solvents were reagent grade and used directly as purchased except for tetrahydrofuran (THF) which was distilled over sodium using benzophenone ketyl as indicator.

All other reagents for synthetic studies were used without further purification unless noted, and were obtained from either Aldrich, Sigma, VWR Scientific, American Scientific Products, Fluka Chemical Corp.

Synthetic Studies

Methyl 7-nitro-4(1H)-quinolone-2-carboxylate 57[74] Six hundred ninety grams (643 mL) of diphenyl ether was heated to vigorous reflux in a 2 L round bottom 3-neck flask. The crystalline dimethyl-(m-anilino)-fumarate **58**[74] (13.8 g, 49.2 mmol) was added in several portions over a 5 min period through a side neck and the reflux maintained for an additional fifteen min. After cooling to room temperature some product had precipitated. Further precipitation was affected by dilution with pet ether (b.p. 35°-60 °C) (750 mL). The 7-nitro isomer was then separated from the 5-nitro isomer by recrystallization from glacial acetic acid, affording pale lustrous yellow crystals which were washed successively with benzene and hexanes (8.30 g, 33.46 mmol, 68% yield). Vacuum sublimation afforded analytical material. mp >300 °C (lit 268-9 °C); ^{1}H NMR (TFA-d_1, 80MHz) δ 9.31(1H, s), 8.80 (2H, m), 8.18 (1H, s), 4.33 (3H, s); ^{13}C NMR (TFA-d_1, 20 MHz) δ 173.26, 162.28, 148.60, 144.34, 142.30, 138.56, 126.84, 125.94, 123.59, 110.59, 57.42; IR (KBr) 3100, 2950, 1740, 1600, 1520, 1350, 1280, 1240 cm^{-1}; EIMS (70eV) 248 (90.0), 188 (100), 114 (38.4); UVmax (c=4.537 x 10^{-5}M, MeOH) 228.2 nm (30,866), 284.6 (17,500), 352.3 (5,080).

Methyl 4-chloro-7-nitroquinoline-2-carboxylate 59 The quinolinone **57** (2.6 g, 10.5 mmol) was heated at reflux in an excess of phosphorus oxychloride (4.82 g, 31.4 mmol, 2.9 mL, 3.0 equiv) for 4 h. The hot reaction mixture was poured over cracked ice (500 g) and stirred for 10 min. The slurry was then neutralized in the cold to pH 9 with concd ammonium hydroxide. Ice was added as necessary to maintain some ice in the mixture during the neutralization. Stirring was then continued until the slurry had warmed to room temperature. The slurry was washed repeatedly with 200 mL portions of dichloromethane until no more particulate matter was present in

the aqueous layer. The combined organic layers were concentrated in vacuo to a volume of 250 mL and filtered through a short bed of silica (Keisel-Gel 60, 1.5 cm x 6.5 cm). Removal of solvent in vacuo afforded 2.5 g (9.4 mmol, 89%) of product as a white powder. This material was normally carried on to the next step without further purification. Recrystallization from hexanes/dichloromethane afforded colorless needles, and vacuum sublimation afforded analytical material: mp 171.5-172.0 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.25 (1H, d, J = 2.1 Hz), 8.52 (1H, dd, J = 9.2, 2.2 Hz), 8.48 (1H, d, J=9.1 Hz), 8.45 (1H, s), 4.13 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.27, 150.15, 149.29, 147.50, 144.46, 130.45, 127.09, 126.28, 123.85, 122.86, 53.77; EIMS (70eV) 266 (5.2), 208 (100), 161 (29.8), 15 (36.1); IR ($CHCl_3$) 1735, 1530, 1440, 1345, 1330 cm^{-1}. UVmax (c=3.086 x 10^{-5} M, MeOH) 290.0 nm (7,160), 259.9 (33,842), 218.7 (27,744); Anal. Calcd for $C_{11}H_7N_2O_4Cl$: C, 49.55; H, 2.65; N, 10.51; Cl, 13.30. Found: C, 49.39; H, 2.57; N, 10.45; Cl, 13.62.

Methyl 7-amino-4-chloroquinoline-2-carboxylate 67 The nitro compound **59** (420 mg, 1.58 mmol) was added to a stirred solution of stannous chloride dihydrate (1.17 g, 5.21 mmol, 3.3 equiv) in concd hydrochloric acid (40mL) at 0°C. Stirring was continued for 4 h while the reaction mixture warmed to room temperature. The resultant orange slurry was then poured over 200 g cracked ice and stirred for 10 min. The pH was adjusted to 9 in the cold by the addition of solid potassium hydroxide pellets. Ice was added as necessary to maintain some ice in the mixture during the neutralization. After warming to room temperature the lemon yellow slurry was extracted with ethyl acetate (3 x 200 mL) and the combined organic layers concentrated in vacuo to 250 mL. The organic layer was then washed successively with 50 mL each of H_2O, 0.1 M NaOH, H_2O, and satd brine. The organic layer was dried over sodium sulfate and reduced in vacuo to afford 320 mg (1.36 mmol, 86%).of product as a lemon yellow powder. Vacuum sublimation afforded anayltical material: mp 200-2 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.94 (1H, d, J = 8.9 Hz), 7.74 (1H, s), 7.27 (1H, dd, J = 8.9, 2.2 Hz), 7.06 (1H, d, J = 2.2 Hz), 6.29 (2H, br s), 3.92 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.74, 151,69, 150.23, 147.37, 141.62, 124.27, 122.26, 118.48, 115.68, 106.46, 52.56; EIMS (70eV) 236 (42.8), 178 (100), 142 (44.4); IR ($CHCl_3$) 3480, 3380, 3150, 1740, 1620, 1580, 1480, 1420, 1360, 1340, 1280 cm^{-1}; UVmax (c=1.804 x 10^{-5} M, MeOH) 401.9 nm (3,642), 293.8 (7,233), 266.0 (44,801), 223.7 (25,657); Anal. Calcd for

$C_{11}H_9N_2O_2Cl$: C, 55.83; H, 3.83; N, 11.84; Cl, 14.98. Found: C, 55.58; H, 3.74; N, 11.52; Cl, 15.01.

Methyl 7-aminoquinoline-2-carboxylate 60 Ten milliliters of an alkaline methanolic solution of potassium hydroxide (40 mg, 0.71 mmol, 1.2 equiv) was added to 30 mL of a methanolic solution of **67** (140 mg, 0.59 mmol) in a 100 mL round bottom flask equipped with a stir bar. The catalyst (25 mg of 10% Pd/C) was added and the mixture stirred under hydrogen atmosphere for 30 min. The catalyst was removed by filtration through celite and the mother liquor reduced in vacuo to afford a yellow-orange residue. The residue was taken up in ethyl acetate (250 mL) and washed successively with 100mL each of 0.1 M NaOH, H_2O, and satd brine. Removal of solvent in vacuo afforded 112 mg (0.55 mmol, 94 %) of product as a yellow powder. Vacuum sublimation afforded analytical material: mp 136-8 °C; ^1H NMR ($CHCl_3$, 400 MHz) δ 8.10 (1H, d, J = 8.3 Hz), 7.93 (1H, d, J = 8.3 Hz), 7.66 (1H, d, J = 8.8 Hz), 7.37 (1H, d, J = 2.2 Hz), 7.08 (1H, dd, J = 8.8, 2.3 Hz), 4.20 (2H, br s), 4.05 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 167.33, 152.56, 151.04, 148.53, 138.58, 129.82, 124.62, 122.78, 117.74, 107.83, 53.36; EIMS (70eV) 202 (49.1), 144 (100); IR (KBr) 3449, 3430, 3329, 1734, 1716, 1639, 1621, 1510, 1465, 1322, 1263, 1109 cm^{-1}; UVmax (c=2.715 x 10^{-5} M, MeOH) 392.5 nm (3,867), 294.0 (6,600), 260.5 (44,287), 217.1 (29,042); Anal. Calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N,13.85. Found: C, 65.10; H, 4.90; N, 13.77.

7-Aminoquinoline-2-carboxylic acid monohydrate 51 The methyl ester **60** (112 mg, 0.55 mmol) was stirred at reflux in 0.1 M sodium hydroxide for 1 h. After cooling to room temperature the mixture was adjusted to pH 6 with 6N HCl. After 1 h the resulting crude precipitate was collected and washed with cold water. Recrystallization from methanol/water afforded 88 mg (0.47 mmol, 84%) of fine orange needles: mp 235-9 °C; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.30 (1H, d, J = 8.2 Hz), 7.77 (1H, d, J = 8.8 Hz), 7.72 (1H, d, J = 8.2 Hz), 7.15 (1H, dd, J = 8.8, 1.9 Hz), 7.11 (1H, s), 6.25 (2H, br s), 5.0 (1H, br s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 165.34, 151.73, 148.32, 147.45, 138.22, 128.77, 121.86, 121.08, 115.47, 104.11; EIMS (70eV) 188 (42.9), 144 (100), 117 (32.5); IR (KBr) 3450-3000, 1640, 1600, 1490, 1420, 1360, 1320, 1240, 1120, 920, 810 cm^{-1}; UVmax (c=4.908 x 10^{-5} M, MeOH) 420.1 nm (4,883), 264.3 (24,350), 255.4 (26,277), 217.1

(30,530); Anal. Calcd for $C_{10}H_{10}N_2O_3$: C, 58.18; H, 4.78; N,13.70. Found: C, 58.23; H, 4.89; N, 13.59.

[4-^{2}H] 7-Aminoquinoline-2-carboxylic acid monohydrate 51a This was produced in two steps from **67** in similar fashion as **51** except that deuterium gas and methanol-d_1 were used in the reductive cleavage step. Thus the chloro amine **67** (1.00 g, 4.23 mmol) was added to a stirred solution of 10% Pd/C (100 mg) and potassium hydroxide (0.26 g, 4.63 mmol, 1.1 equiv) in methanol-d_1 and the mixture stirred for 30 min under deuterium atmosphere. Work-up as for **60** afforded a partially crystalline residue which was subsequently stirred in 100 mL 1N NaOH for 1h. Work-up as for **51** afforded 510 mg (2.70 mmol). of **51a** in 64% overall yield.

Methyl 7-acetamidoquinoline-2-carboxylate Method A The methyl 7-aminoquinoline-2-carboxylate **51** (89.3 mg, 0.44 mmol) was stirred for 3 h in a solution of pyridine (10 mL) and acetic anhydride (2 mL). Solvents were removed as an azeotrope with n-heptane affording a pale brown residue. This residue was dissolved in a minimal amount of 1:1 methanol/ethyl acetate and loaded onto a preparative layer chromatography plate which was eluted with 20% acetonitrile/ethyl acetate. The UV active band was scraped off and eluted thoroughly with ethyl acetate. Concentration of the mother liquor afforded an off-white residue. Recrystallization from methanol/water afforded 78.4 mg (0.32 mmol, 73%) of an off-white powder. Method B The 7-aminoquinoline-2-carboxylic acid **51** (85.9 mg, 0.457 mmol) was stirred overnight in a solution of acetic anhydride (10 mL) and pyridine (20 mL). Solvents were removed in vacuo as an azeotrope with n-heptane to afford a pale brown residue which was dissolved in DMF (5 mL). This residue was treated with methyl iodide (31.3 mg, 0.503 mmol, 31.3 µL, 1.1 equiv) and lithium carbonate (37 mg, 0.503 mmol, 1.1 equiv) and stirred overnight. The reaction mixture was poured into water (50 mL) and adjusted to pH 5 with 1N HCl. This acidic layer was extracted with ethyl acetate (3 x 50 mL), the combined organic layers concentrated in vacuo and the residue purified as described above. No yield. mp 250-1°C (dec) ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.55 (1H, d, J = 1.3 Hz), 8.46 (1H, d, J = 8.3 Hz), 8.01 (2H, m), 7.80 (1H, dd, J = 8.8, 2.0 Hz), 3.95 (3H, s), 2.16 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 169.10, 165.42, 147.89, 147.77, 140.97, 137.05, 128.31, 125.22, 122.31,

119.21, 115.90, 52.33, 24.17; EIMS (70eV) 244 (35.8), 202 (15.6), 186 (23.7), 144 (100); FT-IR (KBr) 3355, 1729, 1688, 1574, 1537, 1490, 1273, 1155, 1040, 870 cm^{-1}; UVmax (c=2.883 x 10^{-5} M, MeOH) 345.9 nm (3,777), 282.2 (8,919), 259.2 (51,698), 211.8 (24,609); High resolution mass spectrum calcd for $C_{13}H_{12}N_2O_3$: 244.08479. Found: 244.08478.

4-Chloro-2,6-dinitrophenol 73 This was prepared from 5-chlorosalicylic acid **71** (10.0 g, 57.9 mmol) by the method of Lin[90b] to afford 8.2 g (37.6 mmol, 65%) of **73**: mp 78-80 °C (lit 79-80 °C); EIMS (70eV) 220 (32.7), 218 (100).

4-Chloro-2,6-dinitroanisole 70 This was prepared from **73** (3.00 g, 1.65 mmol) by the method of Lehmann and McEachern[90d] to afford 2.22 g (9.56 mmol, 70%) of **70**: mp 64-5 °C (lit 65.5 °C); EIMS (70eV) 234 (33.8), 232 (100).

2,6-Dinitrophenol 78 This was prepared by a modification of literature procedure[92]. A stirred mixture of glacial acetic acid (320 mL), concd sulfuric acid (300 mL), and *o*-nitrophenol **79** (200g, 1.44 mol) contained in a 3-neck 2 L round bottom flask fitted with mechanical stirrer and thermometer was heated to 55 °C. A solution of concd nitric acid (145 mL) and acetic acid (30 mL) was added in small portions over a 15 min period while maintaining the temperature between 55 ° and 60 °C. Stirring was continued for 25 min at this temperature. The hot reaction mixtire was poured over cracked ice (500 g) and the precipitate crushed and collected by vacuum filtration. The dinitrophenols were air dried overnight.

A solution of the dinitrophenols and sodium hydroxide (60 g, 1.5 mol) in water (2.5 L) in a 5 L 3-neck flask was heated to 80 C. A hot solution of barium chloride dihydrate (90 g, 0.37 mol) in water (500 mL) was added and the resultant slurry cooled to 35 °C in an ice bath. The precipitate (mostly the barium salt of 2,6-dinitrophenol **78**) was collected by vacuum filtration through a 3 L medium frit sintered glass funnel. The product was rinsed with water (400 mL) and air dried overnight.

The damp precipitate was introduced to a hot solution of sodium carbonate (50 g, 0.47 mol) in water (1 L) and filtered hot to remove precipitated barium carbonate. The filtrate was diluted to 2.5 L, returned to the 5 L flask and heated to 80 °C. The barium salt was again formed by addition of a hot solution of barium chloride dihydrate (67 g, 0.27 mol) in

water (1 L) and the precipitate collected by vacum filtration. The precipitate was added to a hot solution of sodium carbonate (40 g, 0.38 mol) in water (1 L) and hot filtered through a 350 mL medium frit funnel to remove barium carbonate. The product was precipitated out by the addition of 20% hydrochloric acid. The product was collected by filtration, washed with cold water (200 mL) and dried to afford 54 g (0.29 mol, 20.4%) of **78**. A small portion was recrystallized from water affording pale yellow needles; mp 62.5-3.5 °C (lit[92] 63-4 °C).

2,6-Dinitroanisole 74 To a stirred chloroform solution (250 mL) of 2,6-dinitrophenol **78**[92] (10.3 g, 55.9 mmol) in a 500 mL round bottom flask was added methyl iodide (15.5 g, 109.2 mmol, 1.95 equiv) followed by silver oxide (13.8 g, 59.7 mmol, 1.07 equiv). The mixture was stirred at reflux for 4 h. After cooling to room temperature the mixture was filtered through celite and washed with chloroform until the wash was colorless. The mother liquor was reduced in volume to approximately 200 mL and vacuum filtered through a silica bed (6.5 cm x 5.0 cm). The bed was continuously flushed with chloroform until the undesired red chromophore had travelled half way. The mother liquor was reduced to dryness in vacuo to afford a beige powder. Recrystallization from methanol gave 9.8 g (49.5 mmol, 88%) of **74** as colorless needles: mp 118-9 °C (lit[90d] 119 °C); ^{1}H NMR ($CDCl_3$, 80 MHz) δ 8.05 (2H, d, J = 8.2 Hz), 7.35 (1H, t, J = 8.2 Hz).

2-Amino-6-nitroanisole 69a A slurry of 2,6-dinitroanisole **74** (3.0 g, 15.1 mmol) and triethylamine (7 mL, 52.5 mmol, 3.5 equiv) in acetonitrile (6 mL) was added to a 50 mL round bottom three neck flask equipped with a stir bar. The catalyst (100 mg, 10% Pd/C) was then added and the flask fitted with a reflux condenser, addition funnel and stopper. The addition funnel was charged with a solution of 90% formic acid (2.4 mL, 56.2 mmol, 3.7 equiv) in acetonitrile (5 mL). The flask was immersed in a water bath and the formic acid solution added dropwise to the stirred anisole solution over a 10 min period. The addition funnel was replaced with a glass stopper and the water bath replaced with an oil bath. The stirred reaction mixture was heated at vigorous reflux for 5 min while monitoring starting material consumption by TLC (1 hexane:1 ethyl acetate). After cooling to room temperature the reaction mixture was diluted with dichloromethane (10 mL) and filtered

through a celite bed to remove the catalyst. Removal of volatiles in vacuo afforded a red syrup which was treated with cold 20% aq. HCl (30 mL) followed by precipitation of product by addition of concd ammonium hydroxide (20 mL). The crude product was collected and repeatedly digested with portions of hot hexanes. Evaporation of hexanes afforded 2.0 g (11.0 mmol, 78%) of crystalline material: mp 65-7 °C (lit[93,94] 67 °C); ^{1}H NMR ($CDCl_3$, 80 MHz) δ 7.02 (3H, m), 4.05 (2H, br s), 3.85 (3H, s); EIMS (70 eV) 168 (100), 153 (35.2), 92 (45.8).

Dimethyl (2'-methoxy-3'-nitroanilino) fumarate 68 A stirred solution of equimolar amounts (35.7 mmol) of 2-amino-6-nitroanisole **69a** (6.00 g) and dimethylacetylene dicarboxylate (5.12 g, 4.4 mL) in methanol (150 mL) was heated at reflux for 2.5 h. After cooling the yellow crystalline product was collected by filtration and washed sparingly with cold methanol. Recrystallization from methanol afforded 9.33 g (30.1 mmol, 84%) of yellow needles: mp 110-2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.68 (1H, s), 7.52 (1H, dd, J = 8.1, 1.8 Hz), 7.12 (1H, dd, J = 8.1, 8.1 Hz), 7.05 (1H, dd, J = 8.1, 1.8 Hz), 5.65 (1H, s), 3.89 (3H, s), 3.77 (3H, s), 3.75 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.63, 163.82, 146.36, 145.05, 136.37, 125.29, 123.96, 119.68, 96.27, 62.10, 52.97, 51.53, 30.94; EIMS (70 eV) 310 (100), 251 (45.1), 250 (28.3), 219 (86.1); IR ($CHCl_3$) 3300, 2950, 1740, 1680, 1620, 1530,1440, 1360, 1180 cm^{-1}; UVmax (c=1.647 x 10^{-5}M, MeOH) 235.7 nm (13,080), 321.7 (16,778); Anal. Calcd for $C_{13}H_{14}N_2O_7$: C, 50.31; H, 4.55; N, 9.03. Found: C, 50.15; H, 4.45; N, 8.99.

Methyl 8-methoxy-7-nitroquinolin-(4H)-one-2-carboxylate 81 Ninety milliliters of diphenyl ether was heated to near reflux in a 500 mL round bottom 3-neck flask. The crystalline Michael adduct **68** (2.95 g, 9.51 mmol) was added in several portions over a 1 min period through a side neck and the reflux maintained for an additional 2 min. After cooling to room temperature some product had precipitated. Further precipitation was affected by dilution with pet ether (b.p. 35°-60 °C) (250 mL). The yellow gold precipitate was collected and washed with copious amounts of pet ether affording 2.53 g (9.09 mmol, 96%) of product. Vacuum sublimation afforded analytical material: mp 196-7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.43 (1H, s), 8.12 (1H, d, J = 9.0 Hz), 7.75 (1H, d, J = 9.0 Hz), 7.03 (1H, s), 4.14 (3H, s), 4.08 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 178.27, 162.66, 143.65, 142.81, 136.97, 134.43, 128.92,

121.35, 119.19, 113.04, 63.16, 54.13; EIMS (70 eV) 278 (100), 218 (44.7); IR ($CHCl_3$) 3380, 2980, 1735,1640, 1605, 1580, 1525, 1440, 1360, 1280, 1160, 1140, 1100, 1010 cm^{-1}. UVmax (c=3.393 x 10^{-5}M, MeOH) 355.4 nm (7, 413), 226.7 (25,950); Anal. Calcd for $C_{12}H_{10}N_2O_6$: C, 51.80; H, 3.63; N, 10.07. Found C, 51.66; H , 3.48; N, 10.14.

Methyl 4-chloro-8-methoxy-7-nitroquinoline-2-carboxylate 82 A solution of methyl 8-methoxy-7-nitroquinolin-(4H)-one-2-carboxylate **81** (2.53 g, 9.09 mmol) in 15 mL of phosphorus oxychloride was heated at reflux for 4 h. After cooling to 40 °C the warm mixture was poured over cracked ice/water mixture (200 mL) and stirred for 10 min. The solution was then neutralized in the cold to pH 8 by the careful addition of concd ammonium hydroxide. The alkaline solution was extracted with dichloromethane (4 x 250 mL) and the combined organic layers reduced in vacuo to ca. 200 mL. Decolorization and purification were achieved by gravity filtration through a column of aluminum oxide (3.5 cm x 9.0 cm, Baker Reagent Grade, Neutral Powder). Removal of solvent in vacuo afforded 1.98 g (6.69 mmol, 74%) of pure white material. Vacuum sublimation afforded analytical material as a white powder: mp 140-1 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.38 (1H, s), 8.07 (1H, d, J = 9.0 Hz), 8.02 (1H, d, J = 9.0 Hz), 4.49 (3H, s), 4.09 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.33, 151.36, 147.87, 144.47, 143.37, 143.11, 130.46, 124.05, 123.34, 118.94, 64.87, 53.43; EIMS (70 eV) 296 (2.8), 268 (33.0), 266 (100), 234 (37.6); IR ($CHCl_3$) 3520, 1730, 1530, 1340, 1322, 1230, 1150, 980 cm^{-1}. UVmax (c=3.652 x 10^{-5}M, MeOH) 265.6 nm (12,575), 239.2 (25,025); Anal. Calcd for $C_{12}H_9N_2O_5Cl$: C, 48.56; H, 3.06; N, 9.45; Cl, 11.96. Found C, 48.60; H , 2.92; N, 9.48; Cl, 12.19.

Methyl 7-amino-4-chloro-8-methoxyquinoline-2-carboxylate 83 Methyl 4-chloro-8-methoxy-7-nitroquinoline-2-carboxylate **82** (2.0 g, 6.7 mmol) was added in one portion to an ice-bath cooled solution of stannous chloride dihydrate (5.0 g, 22.2 mmol, 3.3 equiv) in concd hydrochloric acid (50 mL). This deep red solution was stirred for 20 h while warming to ambient temperature. The reaction mixture was poured over cracked ice (200 g) and neutralized in the cold with solid KOH pellets. The resultant yellow slurry was extracted repeatedly with ethyl acetate until the aqueous layer was nearly colorless. The combined organic extracts (ca. 1.5 L) were concentrated in vacuo to 500 mL and washed sequentially with 100 mL each

of water, 0.1 N sodium hydroxide, satd sodium bicarbonate, and satd brine. The organic layer was then reduced in vacuo affording 1.5g (5.6 mmol, 83%) of a lemon yellow solid. This material was taken on to next step without further purification. Vacuum sublimation afforded analytical material. mp 148 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.99 (1H, s), 7.84 (1H, d, J = 8.9 Hz), 7.25 (1H, d, J = 8.9 Hz), 4.49 (2H, br s), 4.17 (3H, s), 4.04 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.53, 146.87, 143.60, 143.27, 140.91, 139.32, 121.67, 121.48, 119.80, 117.66, 61.45, 53.00; EIMS (70 eV) 268 (14.7), 266 (42.8), 253 (33.8), 251 (100), 205 (92.7); IR ($CHCl_3$) 3500, 3400, 300, 1720, 1620, 1460, 1440, 1415, 1330, 1220, 1140, 980 cm $^{-1}$; UVmax (c=3.787 x 10^{-5}M, MeOH) 272.0 nm (42,066), 225.6 (22,950); Anal. Calcd for $C_{12}H_{11}N_2O_3Cl$: C, 54.04; H, 4.17; N, 10.51; Cl, 13.29. Found C, 53.91; H, 4.11; N, 10.41; Cl, 13.06.

Methyl 7-amino-8-methoxyquinoline-2-carboxylate 80 The crude methyl 7-amino-4-chloro-8-methoxyquinoline-2-carboxylate **83** (1.0 g, 3.75 mmol) was dissolved in methanol (400 mL) and this solution added to a prepared mixture of KOH (250 mg, 4.50 mmol, 1.2 equiv) in methanol (50 mL). The catalyst (100 mg 10% Pd/C) was added, the flask attached to an atmospheric hydrogenation apparatus and stirred for ca. 30 min until hydrogen uptake had stopped. After removal of catalyst by filtration through celite the mother liquor was reduced to dryness in vacuo. The residue was taken up in ethyl acetate (500 mL) and washed successively with 100 mL each of water, satd sodium bicarbonate and satd brine. The organic layer reduced in vacuo affording a yellow residue. This was purified by flash chromatography (4.0 cm x 14.5 cm) using ethyl acetate as eluent. Reduction in vacuo afforded 725 mg (3.12 mmol, 83%) of a yellow solid: mp 129-30 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.12 (1H, d, J = 8.3 Hz), 7.92 (1H, d, J = 8.3 Hz), 7.45 (1H, d, J = 8.7 Hz), 7.19 (1H, d, J = 8.7 Hz), 4.20 (3H, s), 4.03 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.44, 147.05, 142.46, 139.96, 139.32, 137.13, 124.24, 123.36, 121.01, 117.47, 61.28, 52.80; EIMS (70 eV) 232 (54.8), 217 (100), 171 (90.3), 157 (62.0); IR ($CHCl_3$) 3500, 3400, 3000, 1725, 1630, 1470, 1450, 1380, 1330, 1270, 1220, 1140, 1080, 980 cm^{-1}; UVmax (c=1.554 x 10^{-5}M, MeOH) 399.2 nm (1,415), 267.2 (38,085), 218.4 (22,323); Anal. Calcd for $C_{12}H_{12}N_2O_3$: C, 62.05; H, 5.21; N, 12.07. Found C, 62.29; H, 5.15; N, 12.10.

[4-^{2}H] Methyl 7-amino-8-methoxyquinoline-2-carboxylate 80a This was prepared as described for **80**. Thus the chloro amine **83** (645 mg, 2.43 mmol) was dissolved in methanol-d_1 (300 mL) and this solution added to a prepared mixture of potassium hydroxide (150 mg, 2.67 mmol, 1.1 equiv) in methanol-d_1. The catalyst (240 mg 10% Pd/C) was added and the mixture stirred under deuterium atmosphere. Work-up as described above for **80** afforded 453 mg (1.94 mmol, 80%) of labelled material.

Methyl 8-methoxy-7-trifluoracetamidoquinoline-2-carboxylate 84 A stirred solution of the free amine **80** (200 mg, 0.86 mmol) in pyridine (10 mL) in a 25 mL round bottom flask was cooled in an ice bath. An excess of trifluoroacetic anhydride (1.5 mL) was added and the solution allowed to warm to room temperature while stirred overnight. The solution was diluted with water (10 mL) and extracted with ethyl acetate (1x50 mL). The organic layer was first washed with satd copper sulfate (1x20 mL) and then washed repeatedly with 50 mL portions of water until washings were colorless. The organic layer was concentrated in vacuo to 2-4 mL and precipitation affected by dilution with n-heptane (50 mL). The crude product was purified by flash chromatography (3.5 cm x 12.0 cm) using 1:1 hexane/ethyl acetate to afford 259 mg (0.79 mmol, 92%) of product: mp 149-50 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.97 (1H, br s), 8.69 (1H, d, J = 9.2 Hz), 8.31 (1H, d, J = 8.8 Hz), 8.15 (1H, d, J = 9.2Hz), 7.67 (1H, d, J = 8.9 Hz), 4.49 (3H, s), 4.07 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.66, 154.88 (q, J = 38 Hz), 147.29, 144.39, 140.65, 137.59, 128.60, 128.02, 122.93, 121.40, 120.66, 115.66 (q, J = 289 Hz), 63.48, 53.05; EIMS (70 eV) 328 (12.2), 327 (69.2), 313 (10.8), 312 (79.4), 266 (100); FT-IR (KBr) 3382, 1736, 1526, 1463, 1336 cm^{-1}; UVmax (c=1.554 x 10^{-5}M, MeOH) 259.2 nm (40,123); High resolution mass spectrum calcd for $C_{14}H_{11}N_2O_3F_3$: 328.06709. Found: 328.06700.

[4-^{2}H] Methyl 8-methoxy-7-trifluoracetamidoquinoline-2-carboxylate 84a This was synthesized in identical fashion as that described for **84**. A solution of the free amine **80a** (537 mg, 2.31 mmol) in pyridine (25 mL) was treated with trifluroacetic anhydride (2.5 mL) as described above. Work-up afforded 720 mg (2.19 mmol, 95%) of labelled product.

8-Hydroxy-7-trifluoroacetamidoquinoline-2-carboxylic Acid 85 A sample of **84** (50 mg, 0.15 mmol) was heated to 170 °C in 10 equiv neat

pyridine hydrochloride (176 mg, 1.52 mmol) and maintained for 20 min. The cooled residue was taken up in methanol and reduced in vacuo to afford a red syrup. Addition of water caused the syrup to precipitate. This brown precipitate was collected and air dried affording 26 mg (0.08 mmol, 57%) of crude material; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.22 (1H, s), 10.70 (1H, s), 8.63 (1H, d, J = 8.4 Hz), 8.18 (1H, d, J = 8.4 Hz), 7.75 (1H, d, J = 8.7 Hz), 7.61 (1H, d, J = 8.7 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.93, 155.31, 154.83, 147.49, 144.80, 138.56, 136.73, 128.59, 128.48, 120.10, 119.29, 117.47, 117.18, 114.85; EIMS (70 eV) 300 (33.6), 185 (100); FT-IR (KBr) 3376, 1730, 1688, 1651, 1625, 1540, 1511, 1460 cm^{-1}.

[4-^{2}H] 8-Hydroxy-7-trifluoroacetamidoquinoline-2-carboxylic Acid 85a This was prepared as described above for **85**. Thus the trifluoroacetamide **84a** (400 mg, 1.22 mmol) was heated to 160-70 °C in 10 equiv neat pyridine hydrochloride (1.41 g, 12.2 mmol). Work-up afforded 200 mg (0.67 mmol, 55%) of labelled material.

8-Hydroxy-7-aminoquinoline-2-carboxylic Acid 52 The trifluoroacetamide **85** (500 mg, mmol) was stirred in 0.1 N NaOH (50 mL) for 1 h. The pH was adjusted to 5-6 with 6N HCl and the mixture allowed to stand overnight. The dark purple precipitate was collected by vacuum filtration and air dried overnight. No reliable yield obtained. ^{1}H NMR (DMSO-d_6, 80 MHz) δ 8.30 (1H, d, J = 8 Hz), 7.75 (1H, d, J = 8 Hz), 7.35 (1H, d, J = 9 Hz), 7.25 (1H, d, J = 9 Hz), 3.40 (2H, br s).

[4-^{2}H] 8-Hydroxy-7-aminoquinoline-2-carboxylic Acid 52a The labelled trifluoroacetamide **85a** (200 mg, 0.67 mmol) was stirred overnight at room temperature in 0.1N sodium hydroxide (100 mL). After neutralization with concd HCl the crude **52a** was collected by centrifugation and decantation of the supernatant. The crude precipitate was then repeatedly triturated and resuspended with water, centrifuged and decanted until the supernatant was colorless. The crude product was then immediately treated and used as described in the Feeding Experiments Section.

3-Amino-5-nitroanisole 88 A solution of 3,5-dinitroanisole **90**[117] (9.1 g, 45.9 mmol) and triethylamine (21 mL, 150 mmol, 3.3 equiv) in acetonitrile (150 mL) was added to a 250 mL round bottom three neck flask equipped with

a stir bar. The catalyst (900 mg 10% Pd/C) was then added and the flask fitted with a reflux condenser, addition funnel and stopper. The addition funnel was charged with a solution of 90% formic acid (6.4 mL, 150 mmol, 3.3 equiv) in acetonitrile (20 mL). The flask was immersed in a water bath and the formic acid solution added to the stirred anisole solution over a 30 min period. After stirring for an additional 30 min the reaction mixture was diluted with dichloromethane (100 mL) and filtered through a celite bed to remove the catalyst. The glassware and celite were rinsed well with additional dichloromethane and the organic mother liquor reduced in vacuo to afford a dark brown residue. This was dissolved in sufficient 20% aq. HCl and cooled in an ice bath. Careful addition of concd ammonium hydroxide to pH 8 afforded a crude precipitate. Approximately 3 g of this precipitate was taken up in dichloromethane (75 mL) and washed with water (50 mL). The water layer was then back washed with dichloromethane (50 mL) and the combined organic layers reduced in vacuo. The residue was dissolved in 1% CH_3CN/CH_2Cl_2 (40-50 mL) and loaded onto a silica column (60g, 4.5 cm x 16.0 cm). This was eluted with 1% CH_3CN/CH_2Cl_2 at a flow rate of 50 mL/ 3 min to afford 3-amino-5-nitroanisole (1.5 g) as an orange solid. The remainder of crude product was treated in an analogous fashion (6.5 cm x 20.0 cm column eluted with 1.5% CH_3CN/ CH_2Cl_2) to afford 3.4 g of material. The total combined yield was 64% (4.9 g, 29.1 mmol). mp 123-124 °C (lit[117] 118.5-119.5 °C); ^1H NMR ($CDCl_3$, 80 MHz) δ 3.8 (3H, s), 6.4 (1H, dd, J = 3, 3 Hz), 7.2 (2H, d, J = 3Hz); EIMS (70 eV) 168 (100), 122 (63.6), 107 (31.6), 69 (47.0).

Dimethyl (3'-methoxy-5'-nitroanilino) fumarate 86 A stirred solution of equimolar amounts (20.5 mmol) of 3-amino-5-nitroanisole, **88**, (3.44 g) and dimethylacetylene dicarboxylate, **62**, (2.91 g, 2.52 mL) in methanol (100 mL) was heated at reflux for 5 h. After cooling, the yellow crystalline product was collected by filtration. Recrystallization from methanol afforded pure material (4.4 g, 14.2 mmol). A second crop (1.1 g, 3.55 mmol) was obtained by concentration of the mother liquor to 50 mL and heating at reflux for an additional 18 h. The total yield was 5.5 g (17.7 mmol, 86%): mp 89-90 °C; ^1H NMR ($CDCl_3$,400 MHz) δ 9.70 (1H, s), 7.45 (1H, m), 7.31 (1H, m), 6.71 (1H, m), 5.60 (1H, s), 3.86 (3H, s), 3.80 (3H, s), 3.77 (3H, s); ^{13}C NMR ($CDCl_3$, 100MHz) 169.49, 164.01, 160.64, 149.59, 146.17, 142.22, 112.30, 107.68, 103.82, 97.35, 55.99, 53.19, 51.59; EIMS (70 eV) 310 (53.9) 251 (36.4) 250

(29.7) 219 (100); FT-IR (KBr) 3275, 3110, 2958, 1737, 1674, 1620, 1543,1442, 1352, 1303, 1240, 1206, 1174, 1137, 870 cm^{-1}; UVmax(c=1.3029 x 10^{-5}M, MeOH) 314.0 nm (18,420), 235.2 (18,651), 206.8 (29,933); Anal. Calcd for $C_{13}H_{14}N_2O_7$: C, 50.31; H, 4.55; N, 9.03. Found: C, 50.38; H, 4.41; N, 8.81.

Methyl 7-methoxy-5-nitroquinolin-(4H)-one-2-carboxylate and methyl 5-methoxy-7-nitroquinolin-(4H)-one-2-carboxylate mixture 87a,b Fifty milliliters of diphenyl ether was heated to vigorous reflux in a 250 mL round bottom 3-neck flask. The crystalline Michael adduct **86** (1.73 g, 5.58 mmol) was added in several portions over a 1 min period through a side neck and the reflux maintained for an additional 10 min. After cooling to room temperature some product had precipitated. Further precipitation was affected by dilution with pet ether (b.p. 35°-60 °C) (150 mL). The yellow gold precipitate was collected and washed with copious amounts of pet ether to afford 1.53 g (5.50 mmol, 98% crude yield). The products were not purified or separated but taken directly onto next step. ^1H NMR (DMSO-d_6, 400 MHz) δ 12.14 (2H, br s), 8.35 (1H, d, J = 2.0 Hz), 7.59 (1H, d, J = 2.2 Hz), 7.40 (1H, d, J = 2.0 Hz), 7.35 (1H, d, J = 2.1 Hz), 6.56 (2H, br s), 3.97 (3H, s), 3.96 (3H, s), 3.95 (3H, s), 3.90 (3H, s); ^{13}C NMR (partial), (DMSO-d_6, 400 MHz) δ 176.86, 173.93, 162.06, 161.36, 160.23, 149.28, 148.97, 142.46, 137.87, 111.09, 110.36, 108.69, 102.77, 97.98, 56.41, 56.26, 53.53, 53.36; EIMS (70 eV) 278 (100), 220 (82.2), 218 (42.1).

Methyl 4-chloro-5-methoxy-7-nitroquinoline-2-carboxylate 91a and methyl 4-chloro-7-methoxy-5-nitroquinoline-2-carboxylate 91b The crude product mixture **87ab** (1.15 g, 4.13 mmol) was heated at reflux in phosphorus oxychloride (15 mL) for 4 h. After cooling to room temperature the mixture was poured into an ice/water mixture (50 g) and adjusted in the cold to ca. pH 8 with concd ammonium hydroxide. The mixture was extracted with dichloromethane (4 x 125 mL) and the combined organic layers concentrated in vacuo to afford 1.95 g of crude material. This was dissolved in dichloromethane (200 mL) and impregnated onto silica gel (10 g) by removal of solvent in vacuo. The impregnated silica was loaded onto a wet packed silica column (4.5 cm x 15 cm)and eluted with 60:40 hexane/ethyl acetate to first afford 0.34 g (1.1 mmol, 27%) of the desired methyl 4-chloro-5-methoxy-7-nitroquinoline-2-carboxylate **91a**: mp 166-7 °C; ^1H NMR ($CDCl_3$,

400 MHz) δ 8.17 (1H, s), 7.75 (1H, d, J=2.3 Hz), 7.50 (1H, d, J=2.5 Hz), 4.11 (3H, s), 4.02 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.65, 150.51, 149.03, 146.93, 140.44, 122.63, 118.76, 113.93, 112.20, 56.64, 53.68; EIMS (70 eV) 298 (9.0) 296 (29.7), 240 (31.3), 238 (100); FT-IR (KBr) 3078, 3059, 2954, 1722, 1624, 1541, 1452, 1418, 1327, 1213, 1186, 1115, 952, 869 cm^{-1}; UVmax (c=2.456 x 10^{-5}M, MeOH) 356.4 nm (3,502), 244.4 (39,537), 219.2 (22,476); Anal. Calcd for $C_{12}H_9N_2O_5Cl$: C, 48.56; H, 3.06; N, 9.45; Cl, 11.96. Found C, 48.79; H , 2.91; N, 9.25; Cl, 11.83. Continued elution then afforded 0.77 g (2.6 mmol, 63%) of the undesired methyl 4-chloro-7-methoxy-5-nitroquinoline-2-carboxylate **91b**. mp 202-3 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.78 (1H, d, J = 2.2 Hz), 8.30 (1H, s), 7.74 (1H, d, J = 2.1 Hz), 4.12 (3H, s), 4.11 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.24, 157.41, 149.64, 149.41, 148.89, 143.48, 125.82, 122.80, 119.17, 101.40, 56.66, 53.67; EIMS (70 eV) 298 (13.1), 296 (40.8), 240 (32.1), 238 (100); FT-IR ($CHCl_3$) 1730, 1588, 1537, 1346, 1322, 1231, 1156 cm^{-1}; UVmax(c=1.280 x 10^{-5}M, MeOH) 362.8 nm (391), 278.2 (4,061), 220.4 (5,389); Anal. Calcd for $C_{12}H_9N_2O_5Cl$: C, 48.56; H, 3.06; N, 9.45; Cl, 11.96. Found C, 48.64; H , 2.88; N, 9.16; Cl, 11.46.

Methyl 7-amino-4-chloro-5-methoxyquinoline-2-carboxylate 92a Methyl 4-chloro-5-methoxy-7-nitroquinoline-2-carboxylate **91a** (720 mg, 2.43 mmol) was added to an ice-bath cooled solution of stannous chloride dihydrate (1.81 g, 8.03 mmol, 3.3 equiv) in concd hydrochloric acid (50 mL). This deep red solution was stirred for 4 h while warming to ambient temperature. The reaction mixture was poured over cracked ice (10 g) and neutralized in the cold with solid KOH pellets. The resultant yellow slurry was extracted with ethyl acetate (4 x 250 mL), the combined organic extracts were concentrated in vacuo to 250 mL and washed successively with 0.05 N NaOH (2 x 100 mL), H_2O (1 x 100 mL), and satd brine (1 x 100 mL). The organic layer was concentrated in vacuo and the residue purified by flash chromatography (3.5 cm x 12.5 cm) using ethyl acetate as eluent which afforded 558 mg (2.10 mmol, 86%) of **92a**: mp 188-90 °C; ^1H NMR (DMSO-d_6, 400 MHz) δ 7.53 (1H, s), 6.69 (1H, d, J = 1.9 Hz), 6.67 (1H, d, J = 1.6 Hz), 6.17 (2H, br s), 3.91 (3H, s), 3.88 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.74, 155.92, 152.29, 151.71, 147.23, 140.25, 117.28, 111.47, 101.13, 100.55, 55.81, 52.53; EIMS (70eV) 268(40.9), 266 (93.2), 210 (37.6), 208 (100); FT-IR (KBr) 3455, 3327, 3320, 1729, 1643, 1568, 1470, 1444, 1322, 1279, 1226, 1201, 1144 cm^{-1}; UVmax(c=2.1838 x 10^{-5}M, MeOH) 411.6 nm (3,022), 280.0 (39,884), 233.2

(31,504); High resolution mass spectrum calcd for $C_{12}H_{11}N_2O_3Cl$: 266.04570. Found: 266.04567.

Methyl 5-amino-4-chloro-7-methoxyquinoline-2-carboxylate 92b The 7-nitro-5-methoxy adduct **91b** (400 mg, 1.36 mmol) was treated in an analogous fashion as described above to afford 280 mg (1.05 mmol, 77%) of **92b**: mp 185°C (dec.); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.89 (1H, s), 7.10 (1H, d, J = 2.4 Hz), 6.46 (1H, d, J = 2.3 Hz), 5.21 (2H, br s), 4.06 (3H, s), 3.89 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.03, 161.88, 152.83, 147.23, 144.72, 141.63, 119.80, 112.93, 105.06, 100.71, 55.54, 53.28; EIMS (70eV) 268 (28.2), 266 (88.3), 210 (32.5), 208 (100); FT-IR (KBr) 3455, 3324, 1726, 1631, 1615, 1577, 1446, 1320, 1207, 1151, 1131, 868 cm^{-1}; UVmax(c=2.2218 x 10^{-5}M, MeOH) 403.2 nm (2,386), 286.4 (26,444), 228.8 (33,672), 211.2 (25,524); Anal. Calcd for $C_{12}H_{11}N_2O_3Cl$: C, 54.04; H, 4.17; N, 10.51; Cl, 13.29. Found C, 54.25; H, 4.19; N, 10.32; Cl, 12.41.

Methyl 7-amino-5-methoxyquinoline-2-carboxylate 93a The methyl 7-amino-4-chloro-5-methoxyquinoline-2-carboxylate **92a** (192 mg, 0.72 mmol) was dissolved in of methanol (15 mL) and this solution added to a prepared mixture of potassium hydroxide (48.5 mg, 0.86 mmol, 1.2 equiv) and 10% Pd/C (25 mg) in methanol (15 mL) in a 50 mL round bottom flask. The flask was attached to an atmospheric hydrogenation apparatus and stirred for ca. 30 min until hydrogen uptake had stopped. After removal of catalyst by filtration through celite the mother liquor was reduced to dryness in vacuo. The residue was taken up in ethyl acetate (100 mL) and washed successively with 25 mL each of water, satd sodium bicarbonate and satd brine. The organic layer was then filtered through a small pad of silica and reduced to dryness affording 150 mg (0.65 mmol, 90%) of **93a** as a yellow solid: mp 178-9 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, d, J = 8.4 Hz), 7.65 (1H, d, J = 8.5 Hz), 6.69 (1H, d, J = 1.0 Hz), 6.62 (1H, d, J = 1.0 Hz), 6.04 (2H, br s), 3.94 (3H, s), 3.93 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.93, 155.15, 151.58, 150.22, 147.74, 131.15, 115.13, 114.67, 99.37, 99.19, 55.75, 52.44; EIMS (70 eV) 232 (51.6), 174 (100); FT-IR (KBr) 3441, 3346, 3232, 1732, 1710, 1625, 1572, 1467, 1259, 1199, 1142, 832 cm^{-1}; UVmax(c=2.0208 x 10^{-5}M, MeOH) 406.8 nm (3,315), 272.8 (40,627), 224.8 (25,980), 202.6 (19,299); High resolution mass spectrum calcd for $C_{12}H_{12}N_2O_3$: 232.08479. Found: 232.08477.

[4-^{2}H] Methyl 7-amino-5-methoxyquinoline-2-carboxylate 93c The reaction was carried out as described for **93a** using deuterium gas and methanol-d_1. The chloro amine **92a** (329 mg, 1.24 mmol) was dissolved in methanol-d_1 (25 mL) and this solution added to a prepared mixture of potassium hydroxide (90.2 mg, 1.61 mmol, 1.3 equiv) and 10% Pd/C (110 mg) in methanol-d_1 (10 mL). The mixture was stirred for 15 min under deuterium atmosphere. Work-up as described above afforded 259 mg (1.11 mmol), 90%) of labelled material.

Methyl 5-amino-7-methoxyquinoline-2-carboxylate 93b The amino chloro compound **92b** (114 mg, 0.43 mmol) was reduced in an analogous fashion as described above to afford 100 mg (0.43 mmol, 100%) of **93b** as a yellow foam: mp 159-60 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.18 (1H, d, J = 8.7 Hz), 7.95 (1H, d, J = 8.7 Hz), 7.08 (1H, d, J = 2.2 Hz), 6.53 (1H, d, J = 2.2 Hz), 4.37 (2H, br s), 4.05 (3H, s), 3.87 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.21, 162.10, 150.34, 147.90, 143.22, 130.72, 117.54, 116.41, 103.87, 99.80, 55.53, 53.11; EIMS (70 eV) 232 (79.3), 174 (100); FT-IR ($CHCl_3$) 3357, 3230, 2951, 1734, 1626, 1579, 1442, 1403, 1272, 1213, 1158, 1072, 839, 800 cm^{-1}; UVmax (c=2.4224 x 10^{-5}M, MeOH) 386.0 nm (2,726), 279.2 (26,182), 218.0 (30,972); High resolution mass spectrum calcd for $C_{12}H_{12}N_2O_3$: 232.08479. Found: 232.08477.

7-Amino-5-hydroxyquinoline-2-carboxylic acid hydrobromide 53 The methyl 7-amino-5-methoxyquinoline-2-carboxylate **93a** (150 mg, 0.65 mmol) was heated at reflux in 48% HBr (15 mL) under nitrogen atmosphere for 8 h. After cooling to room temperature the mixture was poured over cracked ice (50 g) and stirred until ice had melted. The hydrobromide salt was collected and washed well with cold water. The solid was air dried overnight to afford 68.7 mg (0.24 mmol, 37% crude yield) of an orange brown powder: mp 280-5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.51 (1H, d, J = 8.1 Hz), 7.61 (1H, d, J = 8.1 Hz), 6.71 (1H, d, J = 1.8 Hz), 6.55 (1H, d, J = 1.7 Hz), 4.10 (?H, br s, D_2O exch). EIMS (70 eV) 204 (34.8), 160 (100). FT-IR (KBr) 3360, 3180, 1673, 1650, 1631, 1577, 1557, 1476, 1441, 1380, 1336, 1292, 1241, 1208, 1159, 1084, 960 cm^{-1}; UVmax (c=3.906 x 10^{-5}M, MeOH) 356.4 nm (2,994), 285.2 (22,760), 225.2 (17,026), 212.8 (22, 760); High resolution mass spectrum calcd for $C_{10}H_8N_2O_3$: 204.05349. Found: 204.05341.

[4-^{2}H] 7-Amino-5-hydroxyquinoline-2-carboxylic acid hydrobromide 53a The labelled **93c** (235 mg, 1.01 mmol) was treated with 48% HBr (20 mL) as described above. After cooling to room temperature the flask was cooled in the refrigerator overnight. The precipitate was collected and dried 12 h in vacuo over phosphorus pentoxide to afford 225 mg (0.79 mmol, 78%) of **53a** as golden needles.

6-Chloro-4-nitro-*m*-cresol 102 This was prepared from *p*-chloro-*m*-cresol, **101**, (1.0 g, 7.0 mmol) by the method of von Walther and Zipper[128] to afford 507 mg (2.7 mmol, 39%) of **102**: mp 134-5 °C (lit 133.5 °C); ^{1}H NMR ($CHCl_3$, 80 MHz) δ10.4 (1H, s), 8.08 (1H, s), 7.04 (1H, s), 2.41 (3H, s); EIMS (70 eV) 189 (31.3). 187 (100).

6-Chloro-2,4-dinitro-*m*-cresol 100 This was also prepared from **101** (25.0 g, 175 mmol) by the method of von Walther and Zipper[128] to afford 33.2 g (143 mmol, 81%) of **100**: mp 70-1 °C (lit 69 °C); EIMS (70 eV) 234 (32.1). 232 (100).

4-Chloro-2,6-dinitro-3-methylanisole 104 This was prepared from **100** (10.0 g, 43.0 mmol) by treatment with methyl iodide (9,2 g, 64.5 mmol, 1.5 equiv) and silver oxide (11.0 g, 47.3 mmol, 1.1 equiv) as described for the synthesis of **69a**[90d,91] to afford 8.4 g (mmol, 79%) of **104**: mp 56-7 °C; ^{1}H NMR ($CHCl_3$, 80 MHz) δ8.05 (1H, s), 4.00 (3H, s), 2.40 (3H, s).

[1-^{14}C] Erythrose 50a This was prepared on a very small scale by minor modification of the method of Serriani, et al.[138] The buffer solutions used were diluted to one-tenth of their original concentration. Thus 0.1 M NaOH and 0.3 M acetic acid were used. The reaction was carried out in a 40 mL conical centrifuge tube using D-glyceraldehyde (11.1 mg, 123 µmol) and [^{14}C] potassium cyanide (8 mg, 123 µmol, specific activity of 8.0 mCi/mmol, 1.25 mCi total). Separation of **50a** from labelled threose **50b** was achieved by collection of 2 mL fractions from a 67 cm x 1.5 cm column of AG50-X8 (Dowex, Ba^{2+} form) with a flow rate of 0.5 mL/min giving threose **50b** (fractions 11-29) and erythrose **50a** (fractions 36-50) in a 10% radiochemical yield. The combined fractions 36-50 were lyophilized and the product dissolved in 10 mL distilled H_2O. The solution was divided into 10 1mL portions and stored in glass vials at 0 °C.

[4-^{15}N] 4-Nitrophthalimide 95 A 25 mL round bottom flask containing 30% fuming sulfuric acid (5 mL) and a stir bar was cooled in an ice bath. To this was added dropwise 99% enriched 50%w/w nitric acid (2 g, 15.6 mmol, 1.5 mL). After 1 min phthalimide (1.25 g, 8.5 mmol) was added and the reaction stirred overnight while warming to room temperature. After a total of thirteen h, the yellow gold solution was poured over cracked ice (25 g) and stirred for ten min. The yellow precipitate was collected by vacuum filtration and washed with water (10 mL). The cake was then stirred with additional crushed ice (25 g), collected, and washed as before. Recrystallization from 95% ethanol afforded 592 mg (3.1 mmole) of product as off white crystals. A second crop was obtained by evaporation of the mether liquor and recrystallization of the residue giving a total yield of 915 mg (4.76 mmol, 56%) of **95**.

[4-^{15}N] 4-Nitroanthranilic Acid Hydrochloride 64a A 25 mL round bottom flask equipped with stir bar and containing a solution of 5.25% NaOCl (368 mg, 5.21 mmol, 7.4 mL) and sodium hydroxide (1.6 g, 40 mmol) in water (10 mL) was cooled in an ice bath. The labelled derivative **95** (9.15 mg, 4.76 mmol) was added in one portion and the solution stirred overnight while warming to room temperature. The cloudy gold reaction mixture was taken to pH 3 by the addition of concd hydrochloric acid and the yellow orange precipitate collected by vacuum filtration and air dried to afford 347 mg (1.58 mmol, 33%) of crude **64a**.

[4-^{15}N] 2,4-Diaminobenzoic Acid Dihydrochloride 45a The anthranilate **64a** (347 mg, 1.58 mmol) was dissolved in methanol (50 mL) in a 100 mL round bottom flask equipped with a stir bar. The catayst (53 mg of 10% Pd/C) and concd hdrochloric acid were added and the mixture stirred under hydrogen at atmospheric pressure for 40 min. Removal of catalyst by vacuum filtration through celite afforded a pale bronxe mother liquor which was concentrated in vacuo to about 10 mL. Precipitation was affected by dilution with ethyl ether (ca 200 mL) to afford 356 mg (1.57 mmol, 99%) of **45a** as a fine red brown precipitate. TLC showed a trace amount of contamination.

2,4-Diacetamidobenzoic Acid A solution of the diamine dihydrochloride **45** (200 mg, 0.89 mmol) in water (10 mL) was treated with

sodium hydroxide (89 mg, 2.23 mmol, 2.5 equiv), sodium acetate (0.22 g, 2.67 mmol, 2.0 equiv), and acetic anhydride (1 mL). After stirring overnight, the white precipitate was collected and washed with water. Recrystallization from water afforded 178 mg (0.75 mmol, 85%) of **111** as white flakes; mp 247-8 °C (lit[143] 260 °C); ^{1}H NMR (DMSO-d_6, 80 MHz) δ11.16 (1H, br s), 10.22 (1H, br s), 8.61 (1H, d, J = 2.1 Hz), 7.92 (1H, d, J = 8.7 Hz), 7.58 (1H, dd, J = 8.7, 2.1 Hz), 2.18 (3H, s), 1.99 (3H, s).

Biosynthetic Studies

General Protocol The microorganism *Streptomyces flocculus* was maintained at 4 °C as spores on agar slants composed of a yeast malt extract prepared according to the following recipe: yeast extract (0.8 g), malt extract (2.0 g), glucose (0.8 g), agar (4.0 g), ddH_2O (200 mL). One slant contains ca 15 mL of this preparation. Seed cultures were prepared by inoculation of 100 mL of a fermentation broth with one-fourth to one-third of a slant via a sterile loop in an Edge GARD™ hood manufactured by The Baker Company, Inc. (Sanford, ME). Fermentations were typically carried out in 1 L flasks containing 250 mL of fermentation broth or in 2 L flasks containing 500 mL of fermentation broth. The recipe for 1 L of fermentation is as follows: glucose (10 g), soybean meal (15 g), distiller's solubles (2 g), $K_2PO_4 \cdot 3H_2O$ (5 g), NaCl (2 g), and $CaCO_3$ (2 g) in 1 L ddH_2O. After sterilization in an AMSCO general purpose steam powered autoclave, the broth was inoculated with 10 mL of the seed culture and shaken in a Lab-Line Model 3595 incubator shaker at 250 rpm and 28-9 °C.

The standard protocol for isolation of streptonigrin was as follows:

After 92 h, the fermentation broths were first vacuum filtered through three layers of cheese cloth to remove the mycelial growth and the mycelial mat pressed to remove excess liquid. If a significant amount of particulate matter was present in the filtrate it was removed by vacuum filtration through celite. The filtrate was rapidly stirred and adjusted to pH 4.75-5.00 by the addition of sufficient 1N HCl. The aqueous solution was then repeatedly extracted with chloroform until no more red color was detectable. Typically 3 x 250 mL extractions were sufficient for a 1 L fermentation. The organic layer was concentrated in vacuo to ca 5 mL and taken up in 0.05 M phosphate buffer, pH 7.5 (25-50 mL) and the remainder of chloroform

removed in vacuo. The aqueous solution was then washed successively with pet ether (bp 35-60 °C, 3 x 50 mL) and isopropyl ether (3 x 50 mL). The aqueous layer was then adjusted to pH 4.75-5.00 as before and extracted with chloroform (2 x 75 mL). The organic layer was reduced in vacuo to afford crude streptonigrin. Hot recrystallization from methanol/THF afforded spectrally pure material.

Biosynthetic Assay Streptonigrin production was determined by Beer's law measuring the UV absorbance of an ethyl acetate solution at 375-80 nm according to the formula:

$$\mu\text{mol STN produced} = \frac{\text{Absorbance at 375-80 nm x dilution}}{0.03798}$$

For the feeding experiments, solutions of precursors were introduced from a syringe through Gelman Acrodisc No. 4192 sterile filters. For trapping experiments, scintillation counting was performed in plastic vials on a Beckmann Model LS7800 Liquid Scintillation Counter on samples dissolved in 10 mL of Beckmann Redi-Solv™ MP scintillation cocktail. Microgram samples were weighed out on a Cahn Model 29 Automatic Electobalance. Culture sonication was performed using a Heat-Systems-Ultrasonics, Inc. Model W-225R cell disruptor set at 50% duty cycle, output control setting 6, for 5 min.

Streptonigrin production was determined by Beer's law measuring the UV absorbance of an ethyl acetate solution at 375-80 nm according to the formula:

$$\mu\text{mol STN produced} = \frac{\text{Absorbance at 375-80 nm x dilution}}{0.03798}$$

Feeding Experiments

[4-^{2}H] 7-Aminoquinaldinic Acid 51a

Trial 1 The free acid **51a** was initially added to ddH_2O and treated with a few drops of 1N NaOH to facilitate dissolution. The solution of the sodium salt of

51a was then distributed evenly amongst 3 x 500 mL fermentations in pulsed fashion over the following schedule:

Time	Amount Fed (volume of solution)
36 h	30.0 mg (10 mL)
48 h	45.0 mg (15 mL)
60 h	48.3 mg (18 mL)

Standard work-up afforded 22.2 mg (43.8 μmol) of STN, **1b**.

Trial 2

Time	Amount Fed (volume of solution)
36 h	45.6 mg (15 mL)
48 h	45.3 mg (15 mL)
60 h	44.3 mg (15 mL)

Standard work-up afforded 13.2 mg (26.0 μmol) of STN, **1b**.

Trial 3

Time	Amount Fed (volume of solution)
28 h	47.0 mg (15 mL)
38 h	44.5 mg (15 mL)
48 h	38.2 mg (15 mL)
58 h	42.4 mg (15 mL)

Standard work-up afforded 20.6 mg (40.6 μmol) of STN, **1b**. Examination of a 10.0 mg (19.7 μmol) portion by ^{2}H NMR showed a singlet at δ8.23 ppm relative to solvent DMSO (δ2.49 ppm) corresponding to H-4 of streptonigrin. Incorporation was determined to be 1.4 %. Spectral parameters were: sweep width = 639 Hz, data points = 2K zero filled to 8K, Hz/pt = 0.16, acquisition time = 1.604 s, pulse width = 45 °, no. of scans = 33 993.

[4-^{2}H] 7-Amino-8-hydroxyquinaldinic Acid 52a This experiment was performed with crude **52a** only. Thus [4-^{2}H] 8-hydroxy-7-trifluoroacetamidoquinoline-2-carboxylic acid **85a** (200 mg, 0.67 μmol) was deprotected by stirring overnight at room temperature in 0.1N NaOH. After neutralization with concd HCl the crude **52a** was collected by centrifugation and decantation of the supernatant. The crude precipitate was then repeatedly triturated and resuspended with water, centrifuged and decanted

until the supernatant was colorless. The wet paste was taken up in ca 80 mL phosphate buffer and a few drops 1 N NaOH added to dissolve all material and maintain a green color. The solution was then administered to 3 x 500 mL fermentations over the identical schedule used in Trial 3 for **51a**. An accurate determination of the amount fed was not calculated but assuming a relatively high yield for the hydrolysis reaction, approximately 100-150 mg were fed. Standard work-up afforded 22.5 mg of STN, **1c**, as determined by UV. Examination of a 12 mg portion by ^{2}H NMR showed no enrichment.

[4-^{2}H] 7-Amino-5-hydroxyquinaldinic Acid 53a This was administered as its sodium salt as described for **51a** over the following schedule:

Time	Amount Fed (volume of solution)
28 h	45.5 mg (15 mL)
38 h	47.4 mg (15 mL)
48 h	45.6 mg (18 mL)
58 h	50.3 mg (15 mL)

Standard work-up afforded 25.1 mg (49.5 μmol) of STN, **1d**. Examination of a 14.6 mg (28.8 μmol) portion by ^{2}H NMR showed no enrichment.

[4-^{15}N] 2,4-Diaminobenzoic Acid 45a This was pulse fed as its sodium salt to 3 x 500 mL fermentations as described for **51a** over the following schedule:

Time	Amount Fed (volume of solution)
24 h	61.1 mg (15 mL)
36 h	20.8 mg (10 mL)
48 h	20.4 mg (10 mL)
60 h	20.2 mg (10 mL)

Standard work-up afforded 37.6 mg (74,2 μmol) of STN, **1e**. Examination of a 22.0 mg (43.4 μmol) portion by ^{15}N NMR showed a singlet at 73,6 ppm relative to external enriched [^{15}N] aniline, 56.5 ppm. Spectral parameters were: sweep width = 2778 Hz, data points = 4K zero filled to 8K, Hz/pt = 0.68, acquisition time = 0.737 s, pulse width = 38 °, line broadening = 2.0 s, relaxation delay = 1.2 s, no. of scans = 22 305. A signal-to-noise ratio approximately 3 times better was achieved employing an INEPTRD sequence

on a 22.5 mg (44.4 μmol) sample. Spectral parameters were: sweep width = 2778 Hz, data points = 8K, Hz/pt = 0.68, acquisition time = 1.475 s, pulse width = 0.0 °, line broadening = 1.0 s, relaxation delay = 0.0 s, no. of scans = 14 959.

[α-^{15}N, α-^{13}C] Tryptophan^{45} 27a/Indole-3-pyruvic Acid 115 Feeding These two compounds were fed simultaneously as their sodium salts prepared as for **51a** over the schedule shown below:

Time	Amount Fed (volume of solution)
37 h	23.7 mg trp, 45.0 mg IPA (24 mL phosphate buffer, 15 mL ddH_2O)
42 h	23.6 mg trp, 44.0 mg IPA (24 mL phosphate buffer, 15 mL ddH_2O)

Standard work-up afforded 29.3 mg (57.8 μmol) of STN, **1f**, as determined by UV. Analysis of a 17.3 mg (34.1 μmol) sample by ^{13}C NMR showed an enhanced singlet at 128.1 ppm but no flanking satellites. Incorporation was determined to be 1.5% using the C-methyl resonance as the normalized reference.

Trapping Experiments

7-Aminoquinaldinic Acid 51 Four 50 mL fermentation broths were each inoculated with 1 mL of a 48 h seed culture in the usual fashion. After 12 h, a total of 13 μCi of [1-^{14}C] erythrose, **50a**, was distributed evenly amongst each of these flasks (resulting in ca 7.15 x 10^6 dpm in each flask). One flask was allowed to incubate for 92 h as a control while the remaining 3 were terminated at a total of 14, 16, and 18 h, respectively.

2 h Trap Authentic **51** (25.1 mg, 134 μmol) was added as carrier to the fermentation immediately prior to work-up. The fermentation was then sonicated for 5 min and the mycelia removed by centrifugation and decantation. The pellet was resuspended in water and the centrifugation, decantation process repeated. The combined aqueous supernatants were lyophilized and the resultant lyophilizate was triturated with methanol and centrifuged. The supernatant was decanted and the pellet resuspended, centrifuged, and decanted. The combined methanolic supernatants were concentrated in vacuo and the final liquid removed under a stream of

nitrogen gas at room temperature. The crude material was dissolved in pyridine (20 mL) and treated with an excess of acetic anhydride (10 mL). After stirring for two days the majority of solvents were removed as an azeotrope with n-heptane followed by azeotrope with dichloromethane. A viscous oil (1.6 g) remained which was dissolved in DMF (10 mL) and treated with 5 equiv each of lithium carbonate (49.3 mg, 0.67 mmol) and methyl iodide (95.1 mg, 0.67 mmol, 41.7 μL). After stirring overnight, the DMF solution was diluted with water to 40 mL and adjusted to pH 6 with 1N HCl. The total activity present at this time was 4.14 x 10^6 dpm (57.9 %). This aqueous layer was extracted with ethyl acetate (3 x 50 mL) and the combined organic layers washed with satd brine (1 x 25 mL). The total activity present in the organic layer was 4.64 x 10^6 dpm (64.9 %). The total activity transfered to the aqueous washes was 0.603 x 10^6 dpm (8.43 %). The organic layer was reduced in vacuo affording a viscous oil (0.95 g) which was taken up in ethyl acetate (4 mL) with a few drops of methanol. This was loaded onto a prep plate and developed with 20% CH_3CN/EtOAc. The blue UV active band was scraped off and the silica eluted with ethyl acetate until no more UV active material was detected. The organics were removed in vacuo and the residue again chromatographed in an analogous fashion. The desired blue UV active band (R_f=0.33) was removed and eluted as previously described. Removal of solvents afforded 11.2 mg of impure acetamido methyl ester. Total activity present prior to recrystallization was 2.90 x 10^6 dpm (40.5 %). To increase the mass available for recrystallization an additional 12.2 mg of authentic acetamido ester was added. Unfortunately, during the first recrystallization a significant loss of material occurred, as only 4.6 mg (18.9 μmol) remained. This material was also quite cold, having retained only 0.67 % of the total activity (2.4 x 10^5 dpm). Subsequent recrystallizations (summarized below) resulted in approximately 50 % loss of total activity per recrystallization. A constant specific activity was never reached, either, as sample dpm approached background.

Recrystallization	Specific Activity(dpm/mmol)	TotalActivity Retained
First	2.4 x 10^5	0.67%
Second	1.99 x 10^5	0.51%
Third	2.25 x 10^5	0.58%
Fourth	1.06 x 10^5	0.27%

Fifth	0.898×10^5	0.23%
Sixth	0.199×10^5	0.07%

4 h Trap In this case no derivatization of **51** was carried out. Initially only 25.3 mg (135 μmol) of authentic carrier was added. Work-up was identical as for the 2 h trap up to concentration of the methanolic supernatant. At this time a total of 5.55×10^6 dpm (77.6%) of the activity remained. Reduction of the methanol solution in vacuo afforded a yellow viscous residue (0.57 g) which by TLC (4 BuOH/1 AcOH/1 H_2O) showed **51** as the dominant product along with two minor impurities. The residue was dissolved in a minimal amount of methanol (ca 10 mL) and passed through a pipet filled with celite to remove insoluble inorganics. The mother liquor was loaded onto a prep plate and eluted with the aforementioned solvent system. The visible bright yellow band was removed and eluted thoroughly with methanol until no more color could be removed from the silica. Concentration of this solution from 400 mL to 50 mL resulted in a large amount of precipitation of inorganics which were removed by gravity filtration through celite as before. At this time an additional 83.7 mg of carrier was added and precipitation affected by concentration to ca 10 mL and dilution with 5 mL water. Recrystallizations from methanol/water (which are summarized below) again afforded cold material.

Recrystallization	Specific Activity(dpm/mmol)	Total Activity Retained
First	5.76×10^4	0.34%
Second	0.729×10^4	0.06%

6 h Trap Work-up in this case was identical to that for the 4 h trap. Initially 34.0 mg (181 μmol) of carrier was added. An additional 59.6 mg (317 μmol) was added prior to inital crystallization. Again a rapid loss of activity occurred.

Recrystallization	Specific Activity(dpm/mmol)	Total Activity Retained
First	3.63×10^5	0.21%
Second	0.092×10^4	0.08%

2,4-Diaminobenzoic Acid Dihydrochloride 45 Four 50 mL fermentation broths (in 250 mL flasks) were each inoculated with 2 mL of a

48 h seed culture in the usual fashion. After 12 h, a total of 13 μCi (3.187×10^7 dpm) of [1-^{14}C] erythrose, **50a**, was distributed evenly amongst each of these flasks (resulting in ca 7.966×10^6 dpm in each flask). One flask was allowed to incubate for 92 h as a control while the remaining 3 were terminated at a total of 14, 16, and 18 h, respectively.

2h trap Authentic carrier **45** (102.2 mg, 0.454 mmol) was added to the fermentation broth immediately prior to work-up. The pH was adjusted to 9.0-9.5 with 1N KOH to dissolve all the added carrier. The fermentation was then sonicated for 5 min and the mycelia removed by centrifugation and decantation. The pellet was resuspended in water and the centrifugation, decantation process repeated. The combined aqueous supernatants were filtered through a bed of celite. The pH of the mother liquor was then adjusted to 5 with 6N HCl causing a large amount of precipitate to form. This was removed by centrifugation in an identical manner as before. The supernatant was lyophilized and the resultant lyophilizate was triturated with methanol and centrifuged. The supernatant was decanted and the pellet resuspended, centrifuged, and decanted. The combined methanolic supernatants were reduced in vacuo and an additional amount of carrier (50.4 mg, 0.22 mmol) added. The residue was dissolved in water (20 mL) and treated with KOH (63.8 mg), NaOAc (0.29 g), and Ac_2O (1mL). After stirring overnight a pale brown precipitate, the bisacetamide, had formed. Further precipitation was affected by acidification to pH 2-3 with concd HCl. The mixture was extracted with ethyl acetate (2 x 50 mL) and the combined extracts concentrated in vacuo to near dryness. The residual liquid was removed as an azeotrope with dichloromethane affording a pale brown powder (26.8 mg). This was repeatedly recrystallized from methanol/water affording material of constant specific activity which retained a total of 0.2% of the total activity fed. The "total specific activity fed" was 1.175×10^7 dpm/mmol. The results are summarized below.

Recrystallization	Specific Activity(dpm/mmol)	TotalActivity Retained
First	2.534×10^5	2.157%
Second	1.070×10^5	0.911%
Third	0.7015×10^5	0.597%
Fourth	0.5081×10^5	0.432%
Fifth	0.2586×10^5	0.220%

Sixth	0.2785×10^5	0.237%
Seventh	0.2329×10^5	0.198%
Eighth	0.2580×10^5	0.220%

4h trap Work-up was performed in an identical fashion to the 2 h trap, initially adding 98.9 mg (0.439 mmol) of carrier **45**. After isolation of the crude material, it was treated with KOH (62.7 mg), NaOAc (0.33 g), and Ac_2O (1mL). In this case only 1.8 mg of crude bisacetamide could be isolated. An additional 20.1 mg of authentic bisacetamide was added prior to recrystallization. This material was quite cold, however, having retained only 0.036% of the total activity fed with a specific activity of only 0.0676 x 10^5 dpm/mmol. The "total specific activity fed" was 1.815×10^7 dpm/mmol. No further work was performed on this trap.

6h trap This trap was worked up in an indentical fashion as described for the 2h and 4h traps. Initially 97.9 mg (0.435 mmol) of carrier was added and an additional 58.5 mg (0.260 mmol) was added prior to acetylation with KOH (70.2 mg), NaOAc (0.26 g), and Ac_2O (1mL) in water (20 mL) as described above. In this case a yellow impurity was present which was not observed in the 2h and 4h traps. This impurity could not be removed by recrystallization and made the recrystallizations cumbersome. In this case a rapid loss of mass occurred, having only 1.8 mg remaining after 3 recrystallizations. The "total specific activity fed" was 1.146×10^7 dpm/mmol. No further work was performed on this material.

Recrystallization	Specific Activity(dpm/mmol)	TotalActivity Retained
First	2.068×10^5	1.80%
Second	1.025×10^5	0.894%
Third	0.294×10^5	0.256%

Acquisition of Two Dimensional ^1H-^{13}C HETCOSY Spectra On STN in DMSO-d_6 Spectral data for **1** were acquired with a sweep width of 20 833 Hz in the F_2 dimension; 128 spectra (256 scans each) were accumulated in 0.2273 msec increments. Resolution was 10.173 Hz/pt in the F_2 dimension and 17.185 Hz/pt in the F_1 dimension.

in TFA-d_1 Spectral data for **1** were acquired with a sweep width of 17 241 Hz in the F_2 dimension; 256 spectra (160 scans each) were accumulated in 0.3125 msec increments. Resolution was 8.419 Hz/pt in the F_2 dimension and 6.250 Hz/pt in the F_1 dimension.

Oxygen 18 Fermentation

A seed culture of *S. flocculus* was prepared in the usual fashion and used to inoclutate four production broths (250 mL in 1 L Erlenmeyer flasks equipped with ball joints). These were connected in series via two sterile filters to a closed system (Fig. 10) containing a burette which was refillable with ^{18}O gas, a small aquarium pump, and a CO_2 trap (aq KOH). Air was circulated at 2 L/min while the fermentation flasks were shaken at 29 °C and 225 rpm. For the first 24 h of the fermentation the burette was charged with $^{16}O_2$ and 0.5 L was consumed. The burette was then filled with the enriched $^{18}O_2$ and over the next 56 h 4.5 L were consumed. For the last 12 h $^{16}O_2$ was again utilized and 1.0 L was consumed. After a total of 92 h, standard work-up afforded 21.1 mg of streptonigrin.

Streptonigrin/$H_2{}^{18}O$ Exchanges

Neutral pH A sample of streptonigrin (20.3 mg, 40.0 μmol) was stirred in the dark in a solution of THF (500 μL) and $H_2{}^{18}O$ (100 μL, 100 mg, 5.0 mmol) for ten h. Solvents were removed under reduced pressure and the residue dried in vacuo. A ^{13}C NMR spectrum (100 MHz, DMSO-d_6) acquired on a 10 mg portion of the treated STN revealed no upfield shifts. Untreated streptonigrin (10 mg) was admixed and another spectrum acquired which again showed no upfield shifts.

Alkaline pH A sample of streptonigrin (21.0 mg, 41.4 μmol) was stirred in the dark in a solution of THF (400 μL), $H_2{}^{18}O$ (100 μL) and satd sodium bicarbonate (100 μL). The resultant solution was pH 10.5. After 16 h the solution was adjusted to pH 4.9 by the addition of three drops of 1N HCl and extracted with chloroform (2 x 10 mL). Reduction in vacuo afforded 20 mg of streptonigrin. A ^{13}C NMR spectrum (100MHz, DMSO-d_6) revealed a ^{13}C-^{16}O resonance at δ 180.26 ppm and an upfield ^{13}C-^{18}O resonance at δ 180.20 ppm for the C-8

carbonyl. No upfield shift was observed for the C-5 carbonyl at δ 175.9 ppm. The peak area integration ratio indicated ca. 30 % exchange.

Acidic pH A sample of streptonigrin (25.1 mg, 49.5 μmol) was stirred in the dark in a solution of THF (200 μL), $H_2{}^{18}O$ (100 μL), and two drops very dilute HCl (ca. 1 x 10^{-4} N) The resultant solution was pH 5.0. After 19 h this was extracted with chloroform (2 x 10 mL) and the combined organic layers passed through a small glass wool plug to remove insoluble materials. After in vacuo removal of solvents, a ^{13}C NMR spectrum, obtained as described previously, showed a resonance at δ 181.31 ppm and an upfield shift at δ 181.27 ppm. The peak area integration ratio indicated ca. 15 % exchange. Again no upfield shift was seen for the C-5 carbonyl.

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