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SPARSOMYCIN – A REVIEW AND RE-ASSESSMENT

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Dedicated to a dear friend and colleague Professor Dr. Somsak Ruchirawat in recognition of his 80th birthday

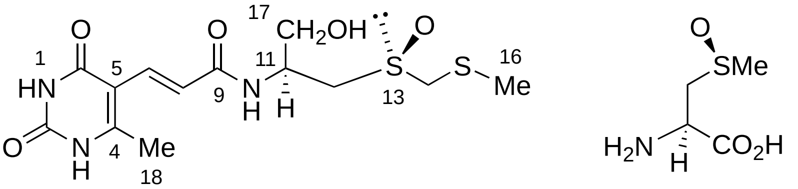
Abstract – The chemistry, biology, and biosynthesis of the microbial alkaloid sparsomycin (**1**) are summarized and re-assessed to identify future research initiatives for this biologically significant metabolite.

INTRODUCTION

One of the underexplored facets of natural product chemistry and biology is the further exploration of "old" bioactive metabolites to fill-in important gaps in basic knowledge, or to explore new or underappreciated applications given the contemporary opportunities in biological assessment and mechanistic understanding. The microbial alkaloid sparsomycin is one such example based on its anticancer, antimicrobial, insecticidal, and tRNA:mRNA translocation activities. Sparsomycin was first reported in 1962 by researchers at the Upjohn Co., Kalamazoo, MI, as a cytotoxic and antitumor alkaloid from the soil microorganism *Streptomyces sparsogenes* var. *sparsogenes,*[1,2] where it co-occurred with tubercidin.[2] Several years later, the molecular formula was corrected to $C_{13}H_{19}N_3O_5S_2$ and the planar structure **1** determined through spectral interpretation and chemical degradation.[3,4] Additional isolations of **1** are rare. For example, a soil sample acquired in Kyoto, Japan, *Streptomyces cuspidosporus* was isolated and culturing yielded sparsomycin (**1**) and the antitubercular alkaloid tubercidin.[5] A water sample from the Nile River afforded **1** from *Streptomyces violaceusniger* AZ-NIOFD,[6] and a derivative of sparsomycin with a unit of H_2O added was reported from a soil sample of *Pseudomonas aeruginosa* AZ-SH-B8 collected in the Sharqia Governorate in northern Egypt,[7] although the characterizations of these isolates were incomplete.

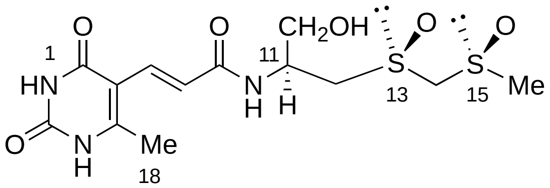
Sparsomycin (**1**) has two stereocenters, the chiral carbon derived from an amino acid moiety and the S_1-sulfoxide unit. The earlier structural studies[2] had established the chiral carbon stereochemistry as corresponding to a D-amino acid however, the sulfoxide stereochemistry was unknown. Their assignments as S_C and R_S were firmly established in 1981.[8] Ottenheijm and colleagues used circular dichroism (CD) as the method to distinguish and assign the S_1-chirality, based on the previous studies of Mislow *et al.*[9] and by Barnsley on *S*-methyl-L-cysteine *S*-oxide (**2**) derivatives.[10] Through X-ray crystallographic analysis of a synthetic intermediate and the sign of its CD curve in the region of 220-230 nm, the sulfoxide was assigned as R_S.[8] This assignment was further supported when the CD curve of each diastereomer was examined. Only one of the four possible stereoisomers is known to occur naturally, access to the other three was the result of total synthesis.[11]

Early biological assessments revealed that sparsomycin (**1**) showed a significant range of effects,[2] including anticancer,[13-15] antibacterial,[5,13-17] antifungal,[1] and antiviral[18] activities, and the ability to inhibit protein synthesis[19-22] through specific A-site and P-site binding characteristics.[23,24]



1 sparsomycin, S_C, R_S

2 *S*-methyl-L-cysteine *S*-oxide



3 sparoxomycin A1, S_C, R_S, S_S

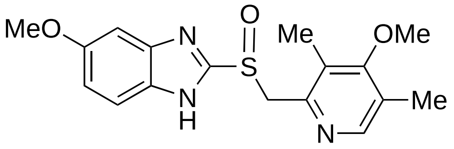
4 sparoxomycin, A2, S_C, R_S, R_S

Figure 1. Structures of sparsomycin (**1**), *S*-methyl-L-cysteine-*S*-oxide (**2**), and the sparoxomycins A1 (**3**) and A2 (**4**)

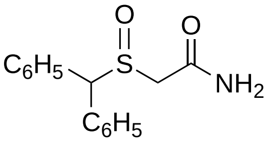
Sparoxomycins Al (**3**) and A2 (**4**) were isolated from *Streptomyces sparsogenes* SN2325 derived from a soil sample collected in Furano-shi, Hokkaido, Japan.[25] The structures were determined as the isomeric S_2-sulfoxide derivatives of **1** through spectroscopic analysis. The stereochemical assignments were deduced through the circular dichroism data, and C-11 was assigned as *S* and S-13 as *R*. The stereochemical differences were at S-15.[26] Biologically, they were inducers of the flat reversion of NRK cells transformed by temperature sensitive Rous sarcoma virus.[25] Sparoxomycin A2 showed weak antimicrobial activity and weak inhibition of protein, RNA, and DNA syntheses.[25]

Synthesis

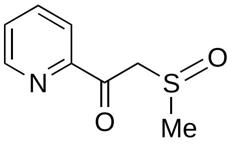
Sparsomycin (**1**) was one of the first natural products characterized to contain a chiral sulfoxide group, and some significant pharmaceutical entities also contain this unit, including esomeprazole (**5**), armodafinil (**6**), oxisurane (**7**), OPC-329030 (**8**), sulindac (**9**), and aprikalim (**10**).[27,28] Progress in the synthesis of chiral sulfoxides, chemically[27-30] and enzymatically[31,32] has been well-reviewed.



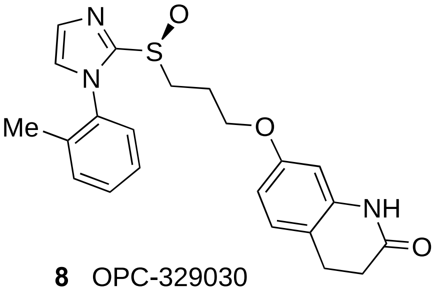
5 esomeprazole



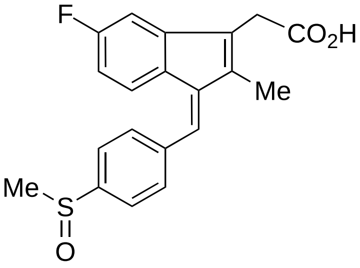
6 armodafinil



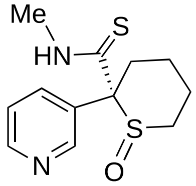
7 oxisurane



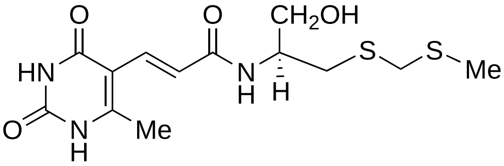
8 OPC-329030



9 sulindac



10 aprikalim



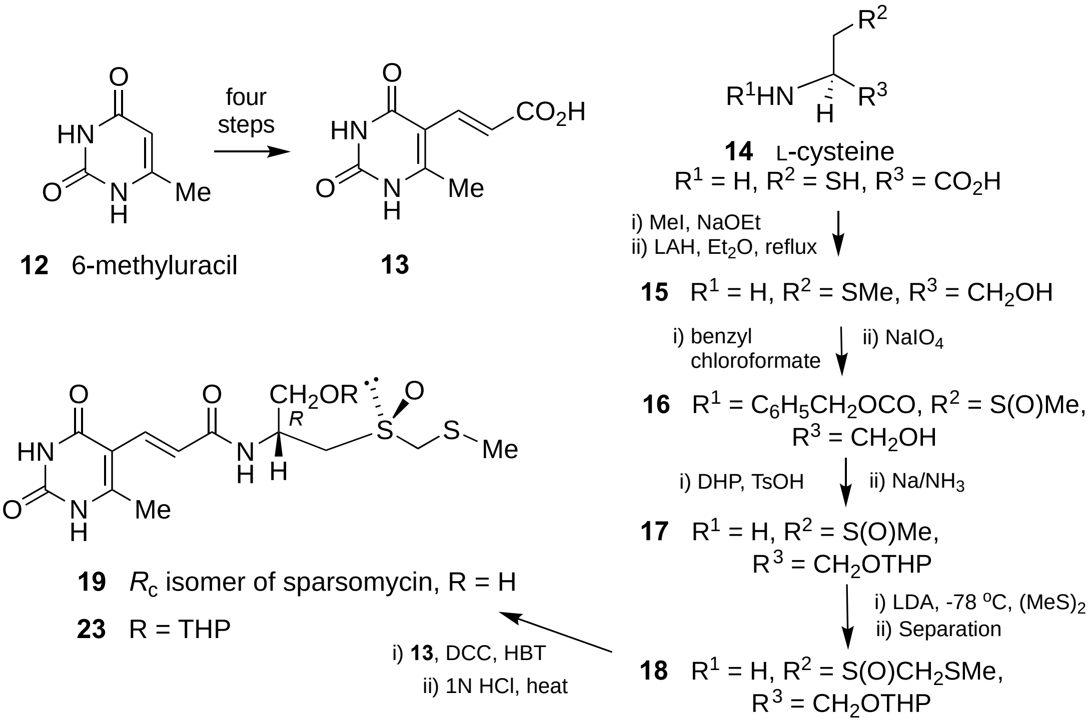
11 *S*-deoxy-L-sparsomycin

Figure 2. Structures of selected sulfoxide-containing pharmaceutical agents and *S*-deoxy-L-sparsomycin (**11**)

Preliminary synthetic studies[33-36] had afforded *S*-deoxy-L-sparsomycin (**11**) and related derivatives,[33] as a prelude to analog exploration for the structure activity relationship studies. Continuing interest in the biological effects of the alkaloid attracted further synthetic approaches to **1** and eventually its diastereoisomers.[11,37-41] These efforts were extended to the development of synthetic routes for analogs for additional biological assessment.[41-47]

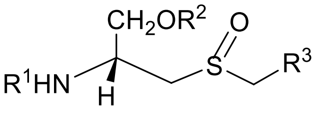
Two groups published their syntheses of the unnatural R_C diastereomer of **1** almost simultaneously,[37,38] in which a convergent approach assembled an acid and an amine to form the amide bond of **1**. Commencing with 6-methyluracil (**12**), the requisite acrylic acid **13**[37] was formed in four steps through hydroxymethylation at C-5, oxidation to the aldehyde, followed by a Wittig condensation with $Ph_3P=CHCO_2C_2H_5$, and base hydrolysis. The methyl dithioacetal sulfoxide moiety was more challenging.

L-Cysteine (**14**) having the *R*-configuration was *S*-methylated and reduced with LAH to yield *S*-methylcysteinol (**15**). Development of the sulfoxide was followed by protection of the alcohol as a THP derivative. Thus, treatment of **15** with benzyl chloroformate and oxidation with periodate afforded the sulfoxide **16** as a 1:1 mixture of diastereomers. Reaction with dihydropyran and reductive cleavage of the *N*-protecting group generated **17**. Chain extension to form the dithioacetal unit was achieved with lithium diisopropylamide (LDA) and dimethyl disulfide to yield the desired **18** whose S_1-isomers were separable. Amide formation in the presence of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HBT), followed by brief treatment with mild acid under reflux to remove the protecting group[38] produced the R_C diastereomer of sparsomycin (**19**) having the opposite optical rotation to the natural isomer (Scheme 1).[37] Note that the *S*-alkylthioacetal group survives these acidic conditions.



Scheme 1. Synthesis of the R_C diastereomer of sparsomycin (**19**)[37]

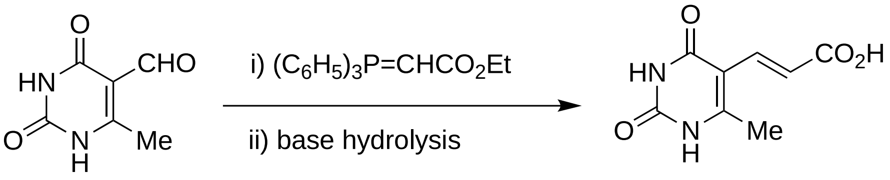
The Ottenheijm approach[38] to an unnatural diastereomer of sparsomycin began with the synthesis of the protected chloromethyl sulfoxide **20** which was converted to the thioacetal **21**.[35] Selective removal of the amine protecting group was achieved in low yield with Na/liq. ammonia and the resulting **22** was coupled with the uracil acrylic acid **13** in the presence of DCC/HBT to afford **23** which was hydrolyzed (0.1N HCl, reflux) to a diastereomer of natural sparsomycin, **19**.[38] Subsequently, the same two groups provided total syntheses of the alkaloid with the natural stereochemistry,[39] and of all four diastereomers[11] which enabled the stereochemistry at both centers in sparsomycin to be defined unambiguously.



	R^1	R^2	R^3
20	$C_6H_5CH_2OCO$	H	Cl
21	$C_6H_5CH_2OCO$	THP	SMe
22	H	THP	SMe

Figure 3. Intermediates in the synthesis of the R_C isomer of sparsomycin (**19**)[38]

Further approaches were developed by Ottenheijm *et al.* to generate the uracil acrylic acid moiety **13** and an appropriate amino acid-derived fragment.[11,36] The preferred route to **13** continued to involve the aldehyde **24**[11] in a Wittig reaction with $(C_6H_5)_3P{=}CHCO_2Et$ and base hydrolysis.

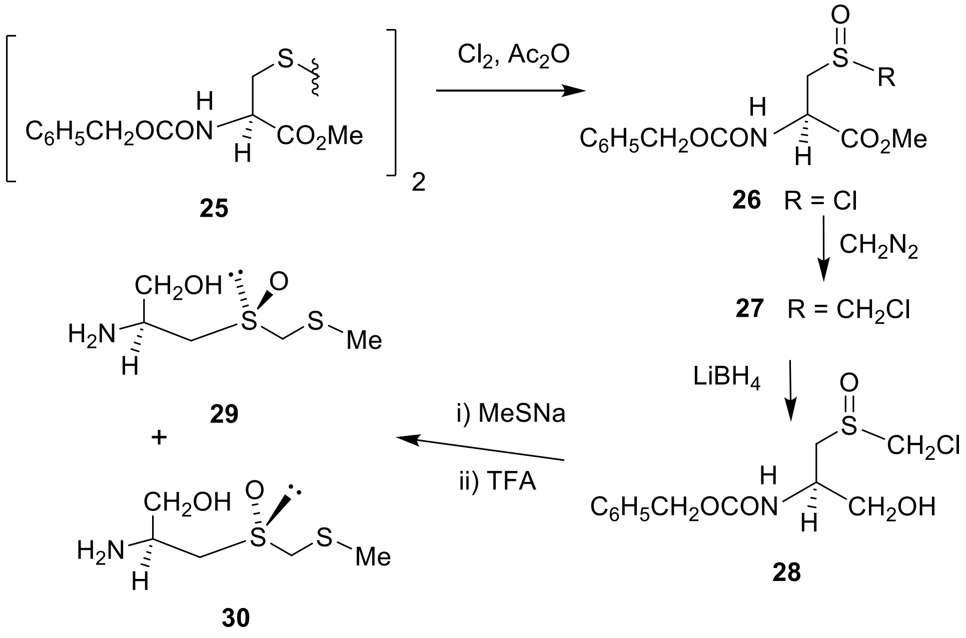


24 **13**

Figure 4. Synthesis of the uracil acrylic acid **13** from the aldehyde **24**

Four potential routes for the transformation of the cysteine thiol group were considered, one involved developing the dithioacetal unit followed by regioselective oxidation. The other routes formed the sulfoxide unit first which could be extended with a -SMe group. Using the Boc-protected methyl ester of D-cystine (**25**), the preferred sequence involved formation of the sulfinyl chloride **26** with chlorine and acetic anhydride which, from reaction with dry diazomethane, yielded the diastereomeric α-chlorosulfoxides **27** (R_C,R_S) and (R_C,S_S). Reduction of the ester moiety with lithium borohydride to the alcohol **28**, thiomethylation, and trifluoroacetic acid hydrolysis of the Boc-protecting group provided the two amines **29** (S_C,R_S) and **30** (S_C,S_S) in 40% overall yield (Scheme 2).[11] Respective coupling of **29** and **30** with **13** afforded natural sparsomycin (**1**) (S_C,R_S) (33% yield) and the S-13 diastereomer **31** (S_C,S_S) (40% yield).

The other two isomers in the R_C-series, **32** and **19**, were prepared from the condensation (DCC/HBT) of **13** and the THP-protected alcohols **33** and **34**, followed by removal of the THP group under mildly acidic conditions to afford **32** (R_C,S_S) and **19** (R_C,R_S). The CD spectra indicated that the S_C,R_S configuration corresponds to sparsomycin (**1**).[11]



Scheme 2. Synthesis of the sulfoxide intermediates **29** and **30**[11]

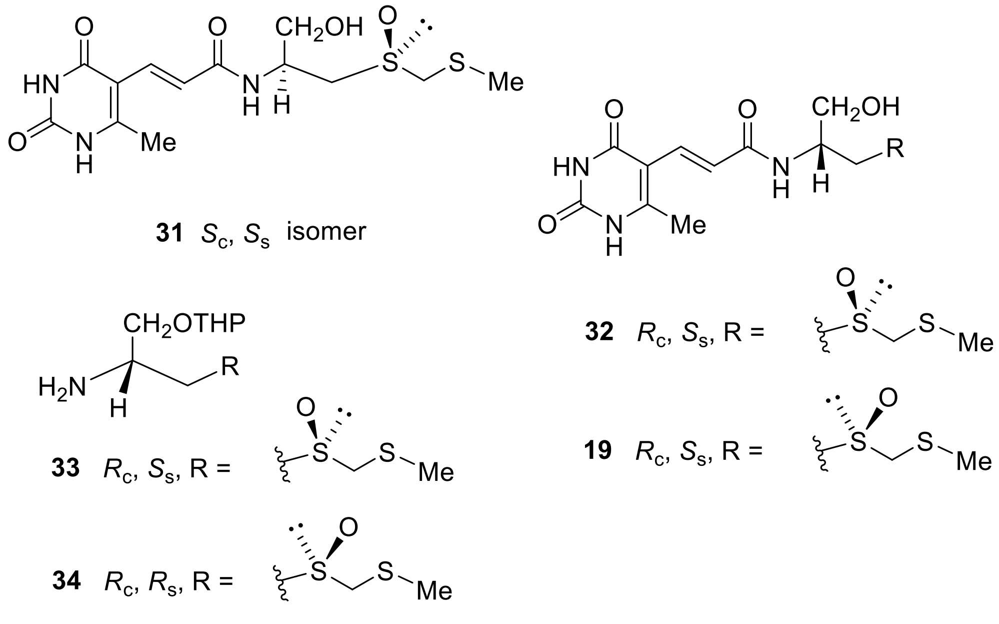
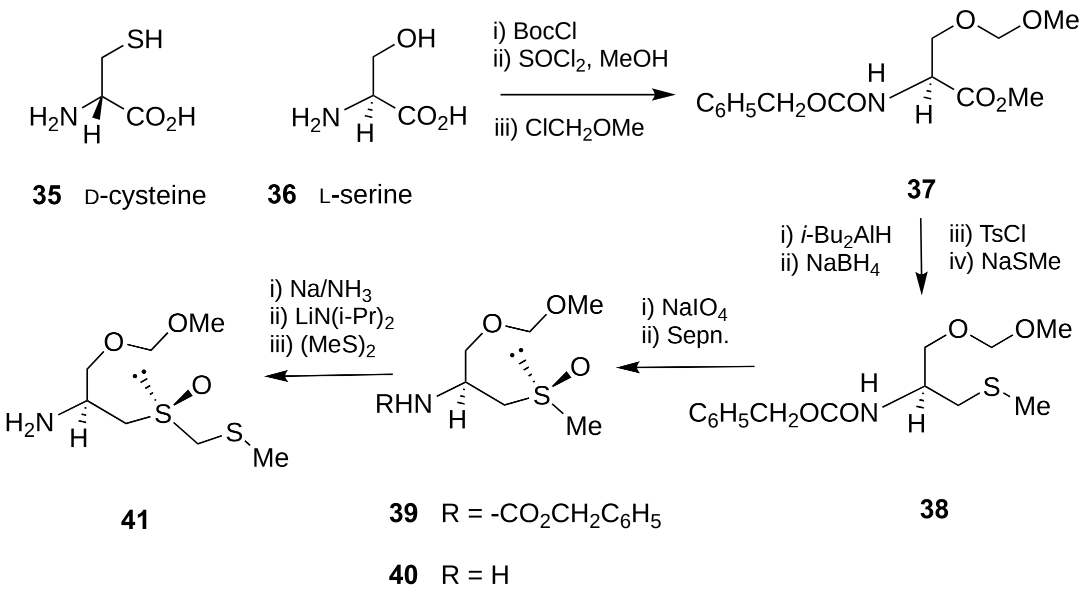


Figure 5. Synthetic intermediates and the target sparsomycin isomers **19** and **32**[11]

Helquist *et al*. focused on developing processes which would afford intermediates in the *S*C series without starting with expensive D-cysteine (**35**) having the *R*-configuration.[39] The elaborate first attempt effectively inverted C-2 of **14** but was deemed too complex for large scale studies. The strategy in the second pathway was to switch the functionalities of the C-2 carbons, thereby inverting the chiral center from *R* to *S*. The route commenced with L-serine (**36**) which was triply protected to afford **37**. Reduction of the methyl ester, tosylation, and thiomethylation produced the methyl sulfide **38**. Oxidation with periodate followed by separation through fractional recrystallization yielded the chiral sulfoxide **39**. *N*-Deprotection (Na/liq. NH_3) to the amino sulfoxide **40** was followed by methylsulfenylation to afford the dithioacetal mono-*S*-oxide **41** (Scheme 3).[39]

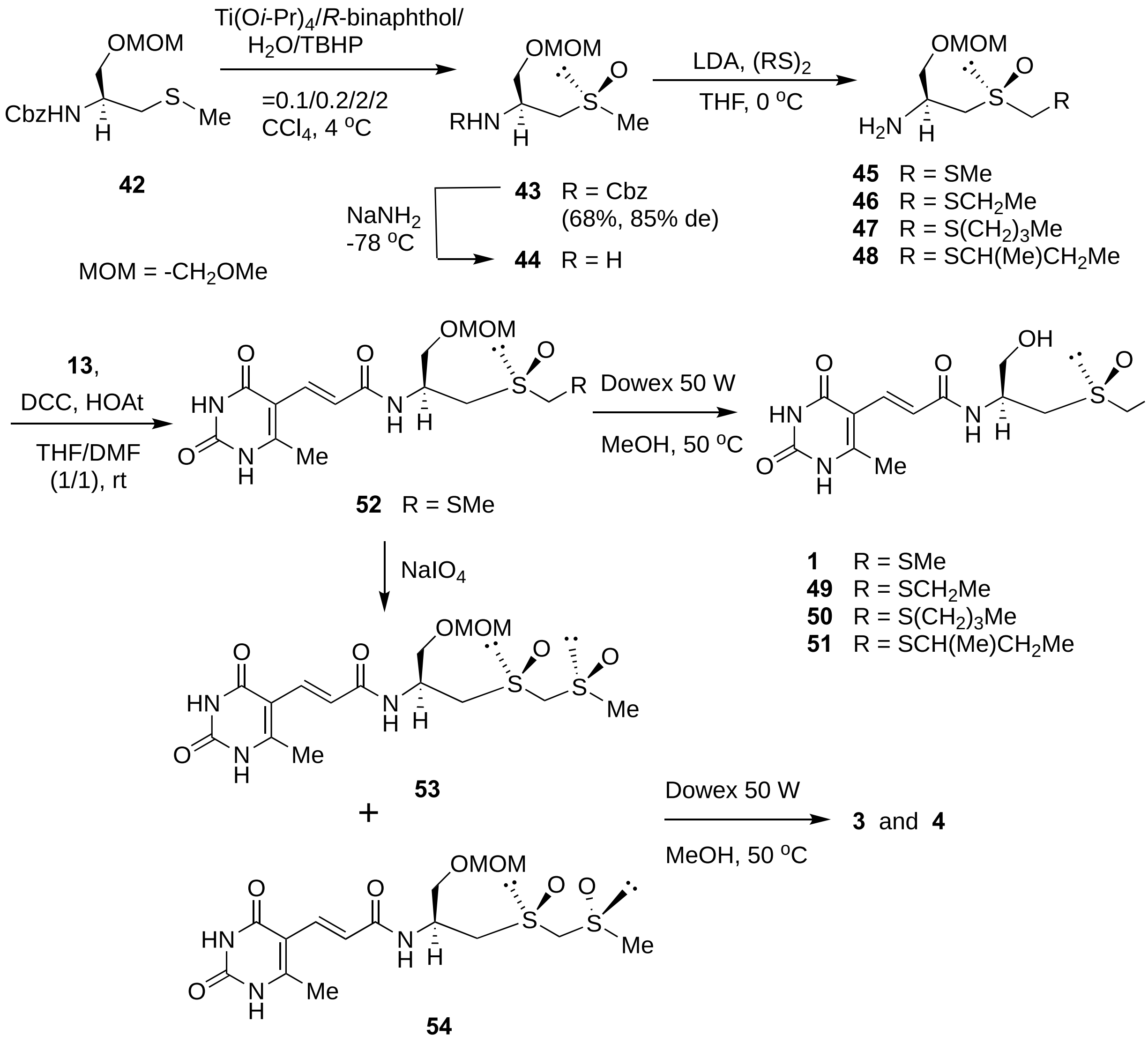


Scheme 3. Synthesis of the monosulfoxide intermediate **41**[39]

A mixed anhydride method generated the amide bond with the uracil acrylic acid **13** and hydrolytic removal of the methoxymethyl group yielded natural (+)-sparsomycin (**1**) having an identical optical rotation to the natural alkaloid.[39]

The structures of the sparoxomycines A_1 and A_2 were supported through a modified synthesis[40] based on the Helquist approach.[37,39] The most significant improvement involved oxidation of the sulfide **42** which was transformed to become a chiral sulfoxide synthesis using a complex derived from $Ti(O\text{-}i\text{Pr})_4$ and *R*-binaphthol.[48,49] This yielded the *S*-configured sulfoxide **43** in 68% yield with 85% diastereomeric excess.[40] Interestingly, the *S*-binaphthol provided no chiral preferences.

Deprotection with Na/liq. NH_3 led to **44** and chain extensions with dialkyl disulfides afforded thioacetyl mono-*S*-oxide derivatives with homologous alkyl groups (i.e., **45**, **46**, **47**, and **48**). Reaction with **13** in the presence of DCC and 1-hydroxy-7-azabenzotriazole (HOAt), followed by deprotection of the alcohol, gave natural **1** and the analogs **49**, **50**, and **51**. Oxidation of the intermediate **52** yielded a 1:1 mixture of **53** and **54** which was hydrolyzed with Dowex 50W in methanol to afford **3** and **4** (Scheme 4).[40] Further improvements in the synthesis of **1** were made subsequently and *S*-alkyl derivatives were developed for biological assessment.[41]



Scheme 4. Synthesis of sparoxomycins A1 (**3**) and A2 (**4**)[40]

Biology and SAR of sparsomycin

Seven structural elements within the scaffold of sparsomycin (**1**) have been probed for their significance to the structure-activity relationships (SAR) in specific biological test systems, i) modulation of the pyrimidine moiety, ii) the stereochemistry of the double bond, iii) the configuration of the amino acid center, iv) the importance of the hydroxy group, v) the presence and stereochemistry of the S_1 sulfoxide, vi) the oxidation level of S_2, and vii) the alkyl group attached to S_2. Aspects of these studies have been reviewed.[45,50] In examining the cytotoxic activity, one of the earliest determinations was that the S_C-configuration, derived biogenetically from a D-cysteine (**35**) moiety, was essential for biological activity,[42] and this conclusion was borne out in subsequent testing.[43,51] Several S_2-alkyl derivatives with a chiral sulfoxide moiety were cytotoxic in the KB system (ED_{50} 1.2 - 2.4 μg/mL) whereas the corresponding sulfides were inactive.[42]

In the competitive inhibition of *N*-acetylphenylalanyl-puromycin formation, the deoxy-S_2-propyl derivative **55** was active in the S_C configuration, and not with the R_C stereochemistry.[43] Removal of the side chain at S_1 eliminated the ribosomal binding activity.[51] The S_2-octyl derivative of sparsomycin **56** had two-fold the cytostatic activity of **1** in the assay for the inhibition of L1210 cancer cells colony formation.[52] When the four isomers of **1** were examined in antibacterial, yeast inhibition, and L1210 colony forming assays, the S_CR_S-configuration of natural **1** was the most active compound.[53] In addition, substituting the CH_2SMe group at S_1 with either -C_3H_7 or -Cl reduced activity, and isomerization of the double bond from *E*- to *Z*- eliminated activity, as did removing the sulfoxide oxygen at S_1. However, the S_2-octyl, -benzyl, and -Et analogs of **1** were more active in the L1210 colony forming assay.[53] Interestingly, against the L5178Y leukemia cell line analogs lacking any sulfur and with a more hydrophobic pyrimidine ring were highly cytotoxic, e.g., **57**, although ED_{50} values were not determined.[54] Broad *in vivo* antitumor testing revealed that **1** was weakly active or inactive, whereas deshydroxy-sparsomycin (**58**), *n*-pentyl-sparsomycin (**59**), and ethyl-deshydroxy-sparsomycin (**60**) were significantly active in the L1210 and renal cell sarcoma models.[55]

Further implications that the peptidyltransferase center where **1** interacts has a hydrophobic core were demonstrated with analogs in which polar groups at the S_2-Me site were inactive, whereas S_2-alkyl group modification produced lipophilic derivatives with excellent activity in the L1210 leukemia assay in mice in which test/control (T/C) >125 for prolongation of life is considered active. The *n*-pentyl derivative **59** showed T/C 386 (at 4.7 mg/kg) and the *n*-heptyl analog T/C 330 (at 6.1 mg/kg) and showed strong activity against *E. coli*.[56] Several other derivatives were more active *in vitro* but were not assayed *in vivo*.

Inhibition of L5178Y cancer cells was examined for *n*-octyl-sparsomycin analogs in which the pyrimidine ring was also modified. IC_{50} values were in the range 1.67-2.72 μg/mL, i.e., about 30-fold less active than **1**. In this series, the sulfoxide configuration and the carbon configuration did not affect the cytotoxicity significantly.[57] The hydroxy group was determined to be non-essential for L1210 colony forming activity. When alkyl groups [e.g., -CH(Me)$_2$, -CH(Me)Et, -$CH_2C_6H_5$] were introduced at this position (C-17) activity was retained while the S_1 sidechain was present.[58] In examining the inhibition of peptide bond formation between puromycin and acetylPhe-tRNA, sparsomycin derivatives in which the S_2-alkyl group was extended were significantly more potent than **1**, most notably the *n*-pentyl **59**, *n*-butyl **61**, and the deshydroxy *n*-butyl **62** analogs.[59]

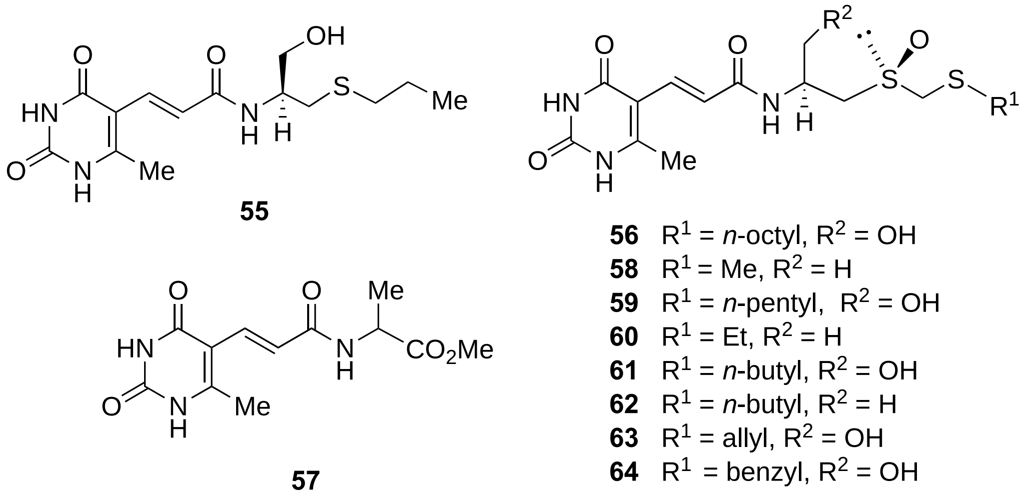


Figure 6. Structures of sparsomycin derivatives for SAR studies[59]

In the morphological reversion assay in *src*^{ts} NRK cells naturally occurring **1** was the most active compound (MEC 6.5 μM), whereas the activity of the homologs **63**, **64**, and **65**, dropped off significantly, and introduction of a sulfoxide group at S_2 as in the alkaloids **3** and **4** eliminated activity (MEC > 530 μM). Derivatives in which the pyrimidinylpropenamide group was replaced with a cinnamoyl group were also inactive.[40] When the four S_2-alkyl derivatives (with R= $-C_2H_5$, $-C_4H_9$, -allyl, and -benzyl) were evaluated in the same assay, the ethyl and allyl analogs were only half as active as **1**, whereas the other compounds were significantly less active.[41] A summary of the structure-activity relationships for the anticancer activity of **1** (NSC-59729) is shown in Figure 7. Chain extension at S_2 increased the hydrophilicity at the carbon chiral center and the pyrimidine ring. Retention of the double bond geometry and the chiral sulfoxide appear to be critical for *in vivo* anticancer activity. However, a clinical evaluation of **1** (i.v. at up to 1 mg/day) against lung, cecum, and colon cancers in advanced patients was halted due to ocular toxicity (blurred vision) from the development of scotomas.[60,61]

The additional biological significance of analogs of **1** became evident when it was shown that through co-administration, ethyl-deshydroxy-sparsomycin (**60**) at 10 mg/kg enhanced the anticancer activity of cisplatin with L1210 cells i.p. in mice 2.8-fold.[62] Sparsomycin (**1**) and the *n*-pentyl **59** and deshydroxy **58** analogs did not potentiate the activity. An extended study with **60** (5 mg/kg) with *cis*-diamminedichloroplatinum(II) (CDDP) (3 mg/kg) produced 80% cures in mice treated with L1210 leukemia cells.[63,64] Further research established that **60** decreased the cellular protein levels and the overall glutathione S transferase activity in opposition to cisplatin which has detoxifying effects.[65] As a single agent, when administered i.p. **60** showed modest *in vivo* activity in mice against the B16 melanoma, good activity (T/C 197%) against the RC carcinoma, and weak or no activity against eight other cancers *in vivo*. No retinotoxic effects were observed in the treated mice.[66]

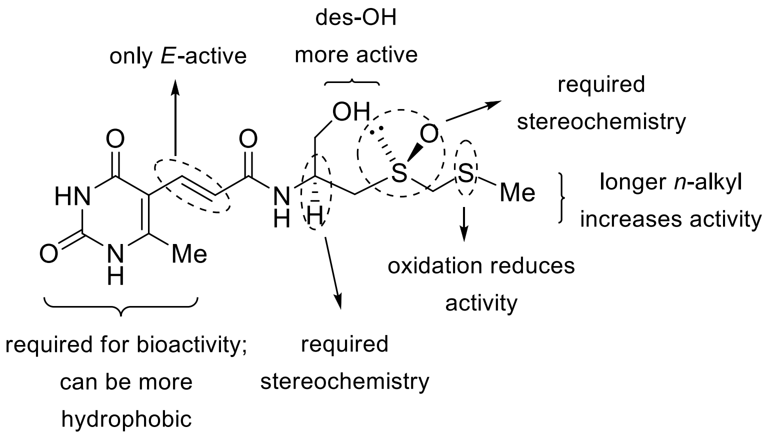


Figure 7. Summary of structure-activity relationships of sparsomycin (**1**) for cytotoxic activity

Sparsomycin and translocation

Every peptide bond that is formed in the chain elongation process of protein synthesis requires that the tRNA:mRNA complex be translocated through the ribosome by three nucleotides. Typically, this is triggered by the presence of elongation factor G (EF-G) and GTP hydrolysis.[67] In this process, three binding sites for the tRNA substrates are recognized which span the ribosomal subunits: the peptidyl-tRNA (P), aminoacyl-tRNA (A), and exit (E) sites. Sparsomycin (**1**) binds at the peptidyl transferase center (PTC) on the large subunit of the ribosome.[50,68] Inhibition of protein synthesis by interfering with the tRNA binding at the A site and the ability to restrict access halts the completion of amide bond formation.[68-74] Characteristically, the uracil moiety stacks with the conformationally altered nucleotide A2602,[70] which is a crucial element for tRNA movement in the ribosome,[71,75] and the hydrophobic sulfoxide tail of **1** limits sterically access to the A site.[76] Sparsomycin (**1**) was one of five antibiotics whose bound relationship to the large ribosomal subunit of the Gram-negative archaeon *Haloarcula marismortui* was evaluated crystallographically at 3.0 Å resolution.[77] Binding of **1** was confirmed to occur primarily at the peptidyltransferase center (P-site) and extended into the active-site hydrophobic crevice (A-site).

In 2003, a completely new facet of the biology of sparsomycin emerged. It was demonstrated that sparsomycin (**1**) could initiate the translocation of tRNAs in the absence of EF-G and GTP.[6] The conclusion was that translocation was an inherent process in the ribosome-tRNA:mRNA complex which could be accomplished with the aid of exogenous small molecules.[6] It was inferred that binding of **1** at the PTC of the 50S subunit of the ribosome was important in the translocation mechanism, which involves the movement of tRNA and mRNA in the 30S subunit about 70 Å away. The rate of translocation triggered by **1** was 900 times faster than the normal background rate, indicating that it is acting at the core of a fundamental process of the ribosome.[6,7]

The promotion of translocation by exogenous substrates is not limited to **1**. Antibiotics which bind to the P-site, such as blasticidin S and erythromycin, do not promote mRNA translocation. However, chloramphenicol, puromycin, and the lincosamides which bind to the 50S A site, do have the same effect, with **1** acting ten times faster and with far greater efficiency. This is possibly due to the more extensive overlap of the tail than the other antibiotics inhibiting both the aminoacyl moiety and the 3'-adenine of the A-site of tRNA.[78] The four sparsomycin isomers and the two chiral carbon stereoisomers at C-11 were examined for their translation ability in the Ac-Phe-tRNA^{Phe} system. Modification of the sulfur-rich tail of **1** and/or changing the chirality at the two centers reduced the ability for translocation, although removing the sulfoxide in the S_C series did not reduce translation; examined separately, the two "halves' of **1** were inactive.[47] Interestingly, when the pseudo-uracil acid moiety of **1** was covalently bound to linezolid analogs, the derivatives **66** and **67** also promote tRNA translocation;[47] linezolid specifically binds to the A site of the ribosome,[79,80] does not promote tRNA translation, and shows some overlap with the binding site of **1**.

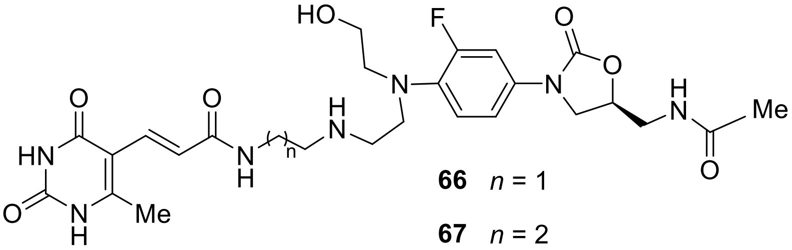


Figure 8. Linezolid analogs **66** and **67** of the uracil acrylic acid **13**[47]

Sparsomycin (**1**) and the linezolid derivatives, but not linezolid itself, could also inhibit the reverse translocation process. It was proposed that the ability to promote the forward translocation, trap the ribosome in the post-translocational state, and inhibit spontaneous reverse translocation is key to the overall effectiveness of the antibiotics.[47,78]

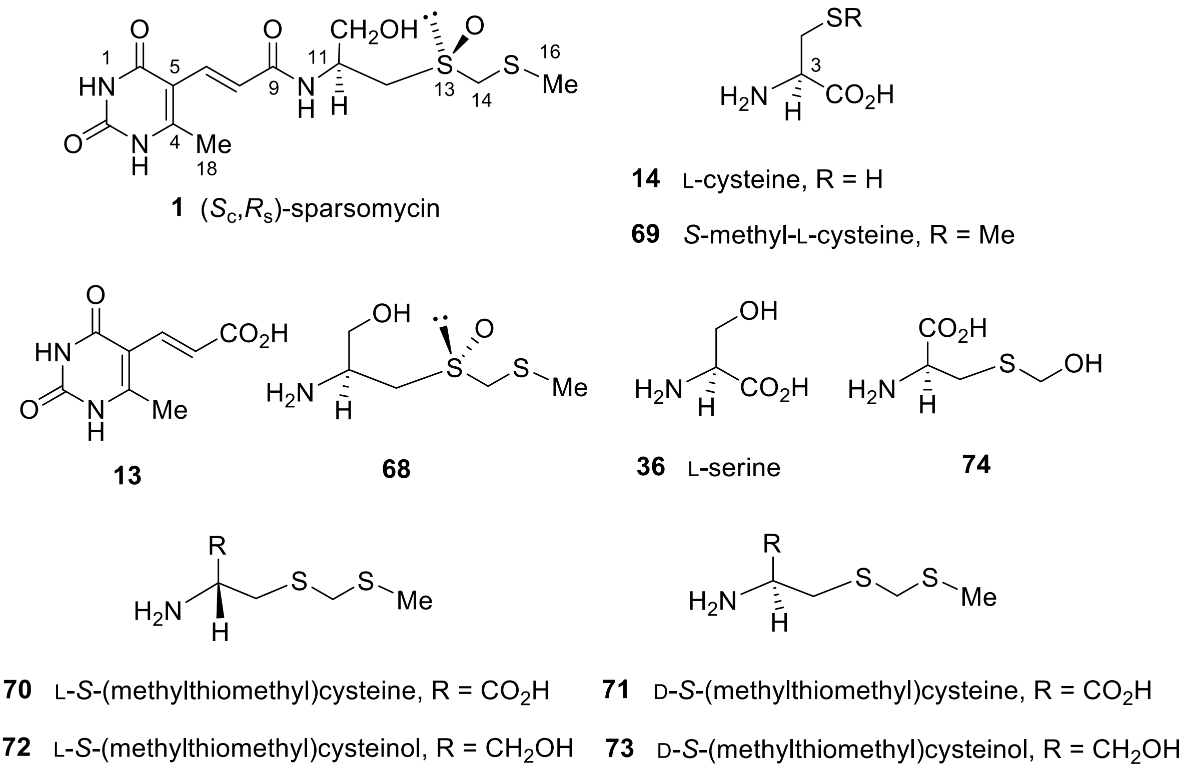
One biological effect of **1** which has been overlooked and not pursued from an analog perspective is the broad insecticidal activity. This was reported in a U.S. Patent issued in 1966,[81] where **1** was claimed to be effective by inducing reproductive sterility in insects through spraying or dusting with **1** at concentrations in the range 0.01 to 1.0% depending on the application. The insects affected included flies, mosquitos, bean beetles, thrips, aphids, mites, salt marsh caterpillars, and army worms, among others.

Biosynthesis

Retrobiosynthetic analysis of sparsomycin (**1**) indicates that the overall key assembly step is the convergent amide formation between a monodithioacetal unit **68** with an uracil acrylate residue **13**. While the derivation of the **68** unit is apparently straightforward based on a postulated elaboration of L-cysteine (**14**), the origin of **13** is biogenetically obscure. Crucial in the pathway is at what stage in the development of the two moieties do they unite, and what is the nature and sequencing of any subsequent tailoring reactions? Parry's group at Rice University initiated exploration of the biosynthetic pathway through labeling experiments to determine the fundamental building blocks.[82,83]

Attention initially focused on examining the origin of the methylene carbons and the methyl groups in **1**. [*methyl*-^{13}C]Methionine was incorporated into two carbons, C-14 and C-16, in the moiety **68**, and not the methyl group (C-18) in the **13** residue or into C-17, suggesting that *S*-methylcysteine (**69**) could be an intermediate.[82,83] Indeed, DL-[3-^{13}C]cysteine (**14**/**35**) was incorporated at C-12, with reduced label appearing at C-14 through an unknown mechanism. The chirality at C-11 corresponds to a D-amino acid and both D- and L-(*methyl*-^{13}C)cysteine (**14**/**35**) labeled C-14.[82,83] It was inferred that introduction of the terminal *S*-methyl group of the **68** unit takes place on **69**. [1-^{13}C]Serine (**36**) labeled C-17 of **1** and [2,3-^{13}C$_2$]serine was incorporated intact into **1** at C-11/C-12, with some fragmentation labeling C-14 and C-16.[83] The timing of the reduction of the carboxyl group of the **14** residue was probed with the L- and D-isomers of [4-^{13}C]-*S*-(methylthiomethyl)cysteine (**70** and **71**) and both were well incorporated.[83] When the reduced forms of **70** and **71**, were examined as precursors, i.e., the corresponding *S*-(methylthiomethyl)cysteinols **72** and **73**, the incorporation rates were much lower, and the D-isomer was preferentially incorporated at C-14. The retention of label at C-17 in **1** from [1-^{2}H$_2$]-**72** and [1-^{2}H$_2$]-**73** suggested that reduction of the carboxyl group derived from **14** precedes coupling with the moiety **13**.[83]

The mechanism of formation of **70** and **71** was postulated to occur through direct sulfur insertion into the *S*-methyl group of **69** followed by *S*-methylation, or through hydroxylation of the *S*-methyl group of **69** to afford a monothiohemiacetal **74**, and subsequent reactions with hydrosulfide and methionine. Both L- and D-(*methyl*-^{13}C)-*S*-methylcysteines were incorporated into **1** at the C-14 position, although in the case of the D-isomer some demethylation and recycling through the one carbon pool occurred with label also appearing at C-16.[83] One complication in accepting this pathway was the unexplained, significant loss of ^{3}H label from [*methyl*-^{14}C, 3-^{3}H]-*S*-methyl-L-cysteine (**69**), presumably from C-12, on incorporation into **1**.



1 (S_C,R_S)-sparsomycin

14 L-cysteine, R = H

69 *S*-methyl-L-cysteine, R = Me

13

68

36 L-serine

74

70 L-*S*-(methylthiomethyl)cysteine, R = CO_2H

71 D-*S*-(methylthiomethyl)cysteine, R = CO_2H

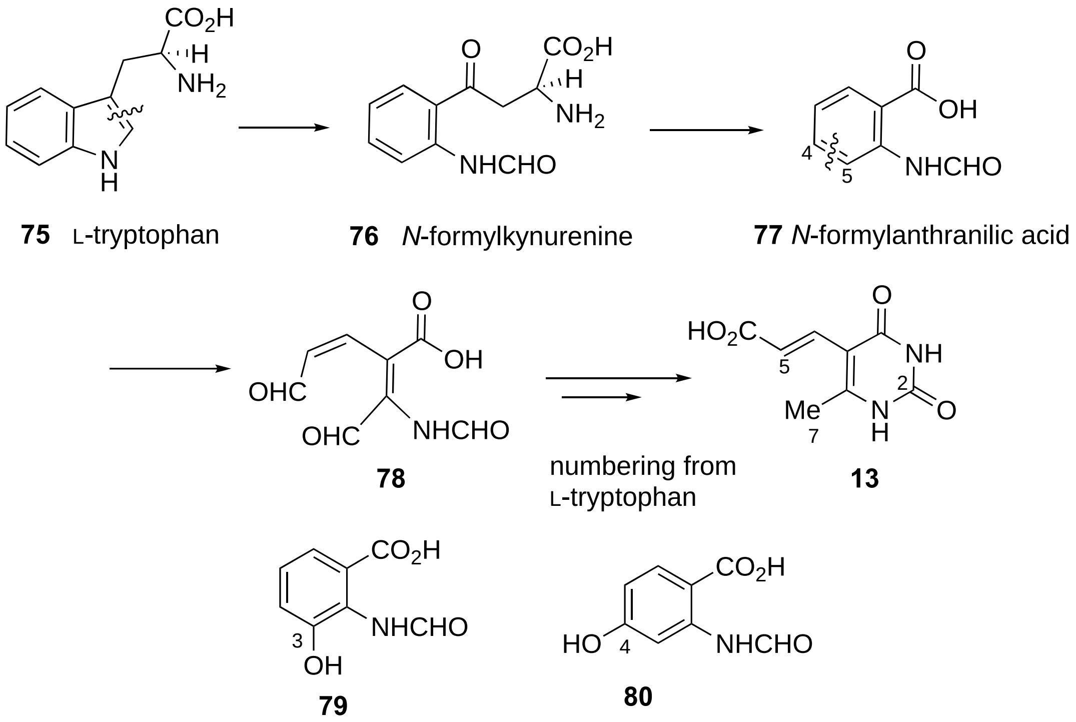
72 L-*S*-(methylthiomethyl)cysteinol, R = CH_2OH

73 D-*S*-(methylthiomethyl)cysteinol, R = CH_2OH

Figure 9. Potential precursors in the biosynthesis of sparsomycin (**1**)[83]

The source of the uracil acrylic acid moiety **13** was postulated to be L-tryptophan (**75**) which could undergo canonical C-2/C-3 cleavage to *N*-formylkynurenine (**76**) and further oxidation (loss of the side chain) to *N*-formylanthranilic acid (**77**). Cleavage at C-3/C-4 would then afford an intermediate **78**. Amination by an unknown source, cyclization, isomerization, and reduction at C-7 (from **75**) produces **13** (Scheme 5).[82,83] In this biogenetic process C-2 of **75** would become C-2 of **1** and C-5 of **1** would be derived from C-5 of **75**. In support of this, [5-^{3}H]-tryptophan (**75**) was incorporated into **1** in *S. sparsogenes*.[82,83] Support for a derivation from **75** also came from the incorporation of DL-[2-^{13}C]tryptophan (**75**) into C-2 of **1**, as predicted, and [5-^{2}H]tryptophan (**75**) was incorporated with retention of label at C-8 in **1**.[82,83] However, an experiment with [5-^{3}H, U-^{14}C]tryptophan (**75**) examining the changing $^{3}H/^{14}C$ ratio led to an ambiguous result not fully supporting the proposed mechanism.[82,83]

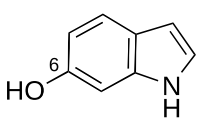
[3,5-$^{2}H_2$]-*N*-Formylanthranilic acid (**77**) yielded **1** with ^{2}H only at C-8, none being retained at C-18.[83] In the proposed pathway, cleavage of the aromatic ring of **77** was projected to occur at C-3/C-4 through hydroxylation at either C-3 or C-4 as a next step. However, neither the 3-hydroxy- nor 4-hydroxy-*N*-formylanthranilic acids, **79** or **80** were incorporated into **1**.[83] Further experiments revealed that *N*-formylanthranilic acid (**77**) was not on the biosynthetic pathway from **75** to **1**.



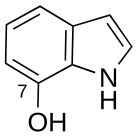
Scheme 5. Biogenesis of the uracil acrylic acid moiety **13** of sparsomycin (**1**)[83]

An alternative pathway was therefore proposed in which ring A of **75** initially undergoes oxidative cleavage at C-6/C-7 through either a 6- or 7-hydroxytryptophan derivative. As shown in Scheme 6, the resulting pyrrole derivative **81** could be reduced at the C-2 carboxyl group to **82** and cleaved between C-4/C-5 to yield **83**. Oxidative deamination and loss of the amino acid side chain would then afford **84** for conversion (amination, oxidation, and cyclization) to **13**.[83]

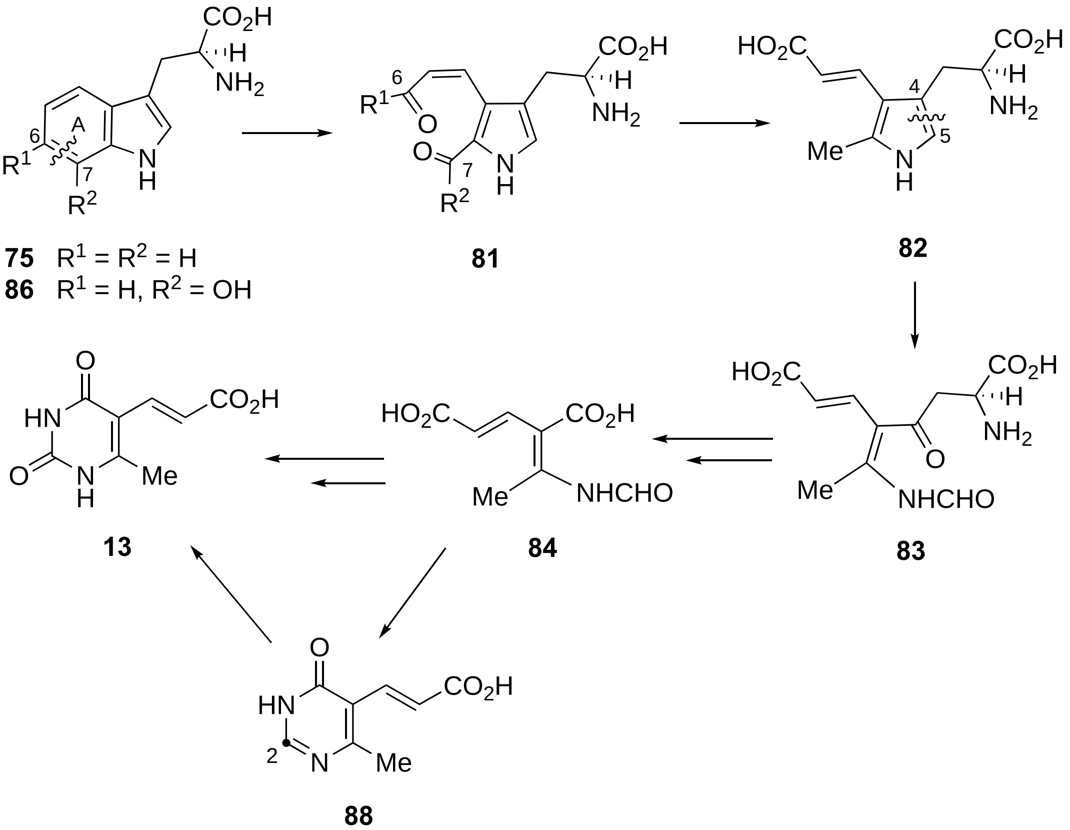
It was recognized that timing of the loss of the side chain of **75** could possibly occur earlier in the biogenetic scheme. In this regard, [6-^{13}C]-6-hydroxyindole (**85**) was not incorporated into **1**, and neither were [2-^{13}C]-7-hydroxytryptophan (**86**) or [2-^{13}C]-7-hydroxyindole (**87**). The last steps in the formation of **13** were investigated with the pyrimidine derivative **88** (hypothetically derived through amination and cyclization of **84**) which was labeled at the C-2 position and was well incorporated into **1**.[83] Subsequently, an NAD$^+$-dependent enzyme for the conversion of (*E*)-3-(6-methyl-4-oxo-5-pyrimidinyl)acrylic acid (**88**) to **13** was purified 740-fold from *S. sparsogenes*.[84,85] In summary, significant progress regarding the precursor units had been made, and aspects of the overall pathways to **68** and **13** developed. However, substantial ambiguity regarding the individual pathway steps and the involvement of specific intermediates remained, although several possible intermediates had been specifically eliminated.[83] The origin of the second pyrimidine nitrogen atom was not investigated in these early studies, and indeed remains of unknown origin.



85 6-hydroxyindole



87 7-hydroxyindole

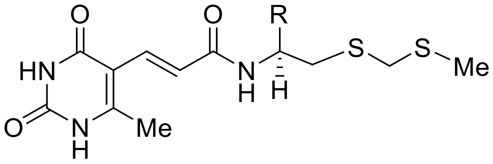


Scheme 6. Alternative biogenesis of the uracil acrylic acid moiety **13** of sparsomycin (**1**)[83]

It was 18 years before further investigations on the biosynthesis of sparsomycin (**1**) were reported, this time at the gene level.[86,87] The genome of *S. sparsogenes* ATCC 25498 was probed through bioinformatics for homologs of xanthine dehydrogenase (XDH) and an NRPS. This search yielded a single gene cluster of ~30 kb and 24 *orfs* (*spaA*-*spaX*). The operon SpsD/SpsE/SpsF was homologous to the heterotrimeric xanthine dehydrogenase[88] and to caffeine dehydrogenase.[89,90] When the genes *spsQ* and *spsR* were assessed for their encoded products, SpsQ was an adenylation-thiolation (A-T) di-domain NRPS, while SpsR had six domains organized as A1-T1-C-A2-T2-E and included the key condensation (C) and epimerization (E) domains envisioned for the biosynthesis of **1**. Mutation of *spsQ* and *spsR* abrogated the formation of **1**.[86] One question was evident immediately from the operon. With only two substrates, why were there three A domains for precursor activation?

After the gene cluster was expressed in *S. lividans* K4-114 as a single plasmid, production of **1** was only observed after L-(methylthiomethyl)cysteine (**70**) was added, suggesting a deficiency for the formation or regulation of **70** in the expressed cluster. SpsQ was highly selective for the activation of **70** and the A1 domain of SpsR was specific for the activation of **13**.[86] Initially, no specific function for SpsR-A2 was determined, although mutant studies indicated that it was essential for **1** formation. As the enzyme with a C domain and an E domain, SpsR was proposed to conduct the condensation of the two carboxyl-group tethered precursors **70** and **13** and to epimerize the L-cysteine-originating moiety. This requires a transthiolation step between the SpsQ-activated **70** and the T2 of SpsR. In support of this pathway, point mutation at the SpsR-T2 domain eliminated the formation of **1**.[86]

Exclusive assembly of **89** was observed from a mixture of **70**, **13**, ATP, SpsQ, and SpsR. Thus, reduction of the cysteine carboxylic acid group occurs *after* condensation and not before, as was suggested earlier.[83] Of four reductases in the cluster, only the addition of the reductase SpsM to the reaction mixture produced *S*C-deoxysparsomycin (**11**) with the hydroxymethyl group at C-11. No intermediate aldehyde derivative was detected.[86] SpsM is a member of the short chain dehydrogenation/reductases requiring NADPH,[91] and without NADPH present no **11** was formed. In the absence of SpsM through mutation, formation of **1** was abrogated, and independently **89** was not a substrate for SpsM. It was therefore viewed as a free-standing reductase which reduces the tethered carboxylic acid residue of the cysteine-derived moiety to the hydroxymethyl moiety and cleaves the amide **11**, *S*C-deoxysparsomycin, from SpsR.[86]



89 R = CO_2H

11 R = CH_2OH

Figure 10. Enzymatic products **89** and **11**[86]

When the only acyltransferase enzyme SpsS in the cluster was added to the SpsQ/SpsR/SpsM system the yield of **11** was enhanced >100-fold, implying that it has a role in the transthiolation processes between the two T domains. At what stage the epimerization step occurs through the action of the E domain in SpsR remains to be determined more precisely. During the transthiolation process is one possibility, or it could take place immediately after the condensation step and before reduction and release of **11**, which has the *S*C geometry of **1**, from the T2 domain.

Thus, 61 years after the discovery of sparsomycin (**1**), the enzymes which unite the two precursor units, **70** and **13**, have been partially functionally characterized, providing a schematic of the closing stages of the pathway. However, the respective roles of the remaining enzymes in the cluster, their substrates, and the reactions which construct the two biosynthetic units **70** and **13** from **14** and **75**, for processing by SpsQ and SpsR, respectively are yet to be delineated.

Conclusions and Future Developments

The intriguing alkaloid sparsomycin (**1**) has been a metabolite of synthetic, biological, and biosynthetic interest for over 60 years. It exhibits a broad range of biological activities, and structure activity relationships (SAR) have been established for the *in vivo* anticancer activity. Clinical experience of a sparsomycin derivative against cecum, colon, and lung cancers indicated retinotoxicity in two of five cases which requires more assiduous investigation as to their evolution, and whether that deleterious effect can be abrogated and separated from the potential anticancer benefits. Analogs which can serve as adjuvants and potentiate the effectiveness or reduce the toxicity of clinical anticancer agents requires further investigation. The enhancement of tRNA:mRNA translocation by sparsomycin (**1**) represents a new avenue to be pursued in terms of SAR *in silico* and through biochemical experimentation. Labeled **1** and the biologically significant derivatives are needed to explore the pharmacokinetics and distribution profile. The *n*-pentyl and *n*-octyl derivatives of **1**, together with their deshydroxy derivatives and the ethyl-deshydroxy derivative, require more detailed assessment in several biological systems (e.g., antiviral, and multidrug resistant antifungal and antibacterial assays). The conjugate analog with linezolid provides a new approach with inhibition at the P- and A-sites occurring due to the selective binding. More examples of conjugates based on molecules which specifically bind to the A-site and which can be united with the uracil moiety are needed. Also of significant interest is the insecticide activity which merits mechanistic investigation and pursuit of SAR. Finally, biosynthetic clarification is important of the enzymes that generate the two biosynthetic units and the precise mechanistic pathways involved. Clarification of the stage at which epimerization occurs on the NRPS SpsR is also needed, together with studies which examine the potential of alternate substrates for the SpsR-SpsQ operon. In summary, sparsomycin (**1**) represents an important opportunity for very interesting chemical, biological, biosynthetic, and possibly clinical explorations.

Conflict of Interest: The authors indicate that they have no conflict of interest.

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