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THE BIOSYNTHESIS OF SINEFUNGIN: INVESTIGATIONS USING A CELL-FREE SYSTEM

Ronald J. Parry* and Shyhchen Ju
Department of Chemistry, Rice University, Houston TX 77251

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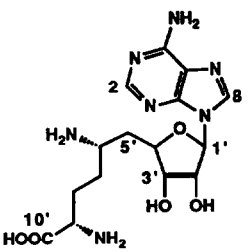
Abstract: The origin of the adenylyl moiety of sinefungin has been investigated by administration of doubly-labeled forms of ATP and adenosine to cell-free extracts of *Streptomyces griseolus*. The results demonstrated that both ATP and adenosine are significantly degraded by the extract before incorporation into sinefungin. However, the ribose ring of adenosine was shown to be incorporated into sinefungin intact, and without loss of tritium from C-5'. This observation rules out the intermediacy of A9145C in sinefungin biosynthesis. Additional experiments are described which suggest that C-C bond formation between C-5 of arginine and C-5 of a ribose derivative may precede attachment of the adenine ring.

Introduction

Sinefungin (**1**) is a novel nucleoside antibiotic isolated from the fermentation broth of *Streptomyces griseolus* and *S. incarnatus*.[1,2] In *S. griseolus*, sinefungin is accompanied by a number of related metabolites including the antibiotic A9145C (**2**). Sinefungin exhibits a variety of interesting properties including antifungal[1]



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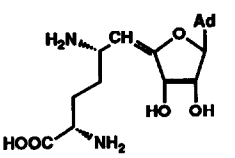

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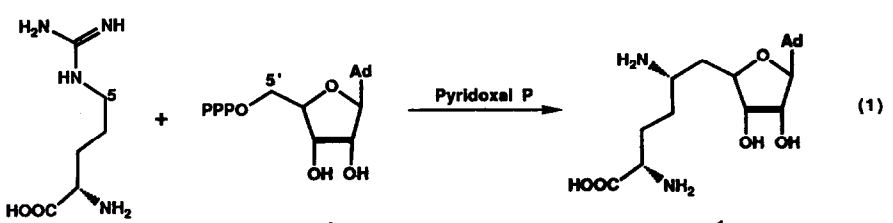
and antiviral[3] activity as well as the ability to inhibit S-adenosylmethionine dependent methyltransferases.[4] Studies of the biosynthesis of sinefungin in cell-free extracts of *S. incarnatus* have been carried out by Robert-Gero and coworkers.[5] The results of these investigations led to the suggestion that sinefungin is biosynthesized from L-arginine (**3**) and ATP (**4**) by a process that requires pyridoxal phosphate (eq. 1). Recent studies in our



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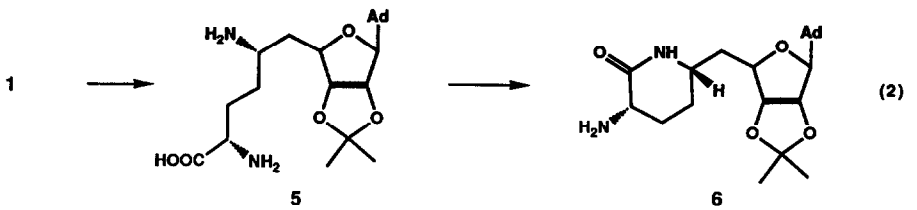
own laboratory employing whole cell cultures of *S. griseolus* have shown that L-ornithine is specifically incorporated into sinefungin with retention of the C-5 nitrogen atom and with overall inversion of configuration at C-5 of the amino acid.[6] These results are completely consistent with the intermediacy of arginine in sinefungin biosynthesis since L-ornithine is the precursor of L-arginine in vivo.[7] We would now like to report the results of investigations whose aim was to clarify the origin of the adenylyl moiety of sinefungin.

Results and Discussion

Cell-free extracts of *S. griseolus* prepared by minor modification of the procedure reported for *S. incarnatus* [5] were found to be capable of synthesizing sinefungin. Preliminary experiments with these cell-free extracts gave results in agreement with previous observations:[5] pyridoxal phosphate and Mg^{+2} were required for sinefungin formation, and radioactive antibiotic was formed from a combination of [U-^{14}C]arginine plus ATP, [U-^{14}C]ATP plus arginine, or [U-^{14}C]adenosine plus arginine. However, the fact that both labeled ATP and labeled adenosine gave rise to labeled sinefungin suggested that the biosynthesis of sinefungin might be more complex than it initially appeared. The role of ATP and adenosine in sinefungin biosynthesis was therefore evaluated with the aid of doubly-labeled precursors.

[5'(*RS*)-5'-^{3}H]-2',3'-O-Isopropylideneadenosine was synthesized from 2',3'-O-isopropylideneadenosine by the procedure reported for the preparation of the corresponding deuterated compound.[8] The labeled nucleoside was then converted into [5'(*RS*)-5'-^{3}H]ATP using the chemistry devised by Moffatt.[9] The tritiated ATP was mixed with [8-^{14}C]ATP and the doubly-labeled ATP was incubated with arginine and pyridoxal phosphate in a cell-free extract from *S. griseolus*. Sinefungin was isolated by isotope dilution and rigorously purified by conversion to its isopropylidene derivative (**5**) and then to the corresponding isopropylidene lactam (**6**) (eq. 2).

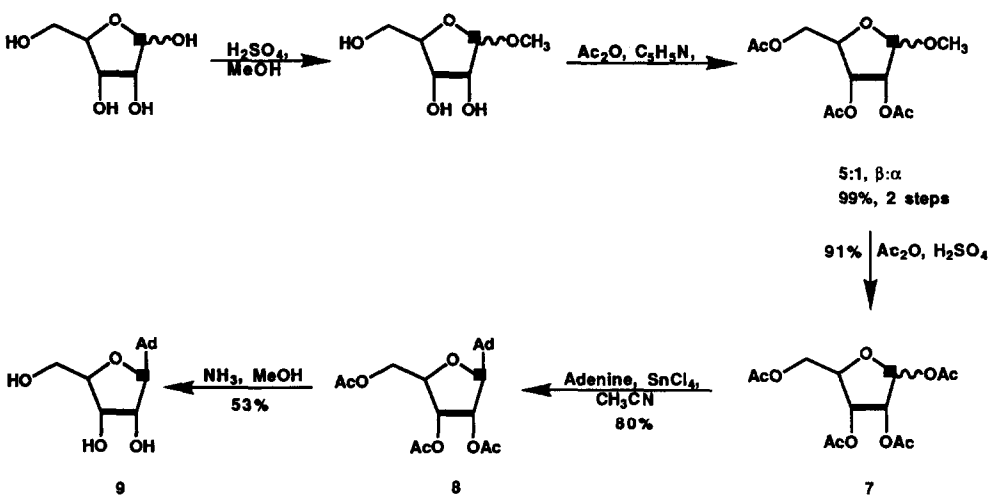


The isotopic ratio in the purified sinefungin (Table 1, expt. 1) indicates that ATP is not incorporated intact into sinefungin by the cell-free extract, but is broken down into labeled fragments that are then incorporated. The catabolism is presumably due to the presence of a nucleoside hydrolase or nucleoside phosphorylase in the extract.[10] Additional evidence in favor of this interpretation was obtained by examining the incorporation of [5'-^{3}H]ATP into sinefungin in the presence of 8-amino-2'-nordeoxyguanosine, a potent inhibitor of purine nucleoside phosphorylase.[11] In this experiment, the incorporation level of the labeled ATP into sinefungin dropped 20-fold relative to a control incubation carried out in the absence of inhibitor (Table 1, expt. 2). These results are consistent with the thesis that ATP is incorporated into sinefungin after an enzyme-catalyzed cleavage of the glycosidic linkage. However, we cannot rule out the possibility that the drop in incorporation level may be at least partly due to inhibition of the sinefungin forming enzyme(s) by the inhibitor.

The role of adenosine as a sinefungin precursor was evaluated using two forms of doubly-labeled adenosine. Initially, [5'(*RS*)-5'-^{3}H]adenosine was prepared from the corresponding isopropylidene derivative and mixed with [U-^{14}C]adenosine. The doubly labeled adenosine was converted to sinefungin using the cell-free extract, and the sinefungin was isolated and purified in the usual fashion. The isotopic ratio exhibited by the purified antibiotic (Table 1, expt. 3) was much closer to the expected value than in the case of ATP, but the discrepancy was sufficiently large to suggest that the adenosine was also being catabolized by a nucleoside hydrolase or nucleoside phosphorylase present in the extract. Incubation of [5'(*RS*)-5'-^{3}H, U-^{14}C]adenosine and 8-amino-2'-nordeoxyguanosine with the cell-free extract resulted in a 20 fold reduction in the incorporation level for this nucleoside (Table 1, expt. 4). This observation can be interpreted in the same manner as the results obtained using this inhibitor and ATP. It is noteworthy, however, that the labeled sinefungin isolated in this experiment showed no improvement in the tritium to carbon-14 ratio in comparison to experiment 3. This indicates that the small amount of radioactivity found in the sinefungin isolated in experiment 4 still arises by breakdown of the labeled adenosine.

The obvious lability of the C-N glycosidic linkage suggested that a clearer result would be obtained if adenosine doubly labeled in the ribose moiety was employed as a precursor. Therefore, [1'-^{14}C]adenosine (**9**) was synthesized from commercial [1-^{14}C]-D-ribose by the procedure outlined in Scheme I. [1-^{14}C]-D-ribose was

Scheme I



converted in high yield to [1-^{14}C]-1,2,3,5-tetra-O-acetyl-D-ribofuranose (**7**) using chemistry reported by Guthrie et al.[12] This two step procedure for the preparation of **7** is much more efficient than the direct acetylation of D-ribose, since the latter leads to the formation of significant quantities of the ribopyranose tetracetate.[13] The labeled ribofuranose derivative **7** could be smoothly converted into [1-^{14}C]-2',3',5'-O-triacetyladenosine (**8**) by coupling with adenine in the presence of stannic chloride.[14] Deprotection of **8** with methanolic ammonia then gave [1'-^{14}C]adenosine (**9**) with an overall yield of about 38%. Incubation of a mixture of [1'-^{14}C]- and [5'(*RS*)-5'-

^{3}H]adenosine with the cell-free extract yielded sinefungin that exhibited the same ratio as the precursor, within experimental error (Table 1, expt. 5). This result clearly demonstrates that the ribose moiety of adenosine is incorporated intact into sinefungin, and it also shows that no tritium is lost from the C-5 position of the ribose moiety as the result of C-C bond formation to arginine. This rules out the possible intermediacy of A9145C in sinefungin biosynthesis.

Further evidence that sinefungin can be generated in the cell-free extract from the fragments of ATP or adenosine catabolism was procured in the following ways. Administration of [1-^{14}C]-D-ribose to the extract in the presence of arginine, ATP, and pyridoxal phosphate yielded radioactive sinefungin exhibiting the highest incorporation figure thus far observed (Table 1, expt. 6). Ribose therefore appears to be a more efficient precursor of sinefungin than either adenosine or ATP. Administration of a combination of [1-^{14}C]ribose and [8-^{3}H]adenine to the extract in conjunction with arginine and pyridoxal phosphate generated doubly labeled sinefungin (Table 1, expt. 7), thereby demonstrating that the adenylyl moiety of **1** can be assembled from ribose and adenine in this cell-free system.

Table 1. Incorporation of Precursors into Sinefungin by Cell-free Extracts of *S. griseolus*[a]

Expt. No.	Precursor (^{3}H/^{14}C)	% Incorpn.	^{3}H/^{14}C (% ^{3}H retention)
1	[5'(*RS*)-5'-^{3}H, 8-^{14}C]ATP (5.50)	0.05	14.4 (262)
2	[5'(*RS*)-5'-^{3}H]ATP plus 8-amino-2'-nordeoxyguanosine (3.7 mM)	0.001	---
	[5'(*RS*)-5'-^{3}H]ATP (control)	0.02	---
3	[5'(*RS*)-5'-^{3}H, U-^{14}C]adenosine (5.80)[b]	0.10	4.50 (78)
4	[5'(*RS*)-5'-^{3}H, U-^{14}C]adenosine (6.55)[b] plus 8-amino-2'-nordeoxyguanosine (7.4 mM)	0.005	4.50 (69)
5	[5'(*RS*)-5'-^{3}H, 1'-^{14}C]adenosine (8.00)[b]	0.08	7.92 (99)
6	[1-^{14}C]-D-ribose[c]	1.1	---
7	[1-^{14}C]-D-ribose, [8-^{3}H]adenine (1.32)[b]	0.07	0.83

[a] All incubations were carried out for 80 min. [b] ATP was absent from this incubation. [c] ATP was present in this incubation.

The results of the investigations outlined thus far suggested that the adenylyl moiety of sinefungin might be assembled *after* C-C bond formation between C-5 of arginine and C-5 of a ribose derivative. A series of experiments was therefore carried out to evaluate this hypothesis. [1-^{14}C]-D-Ribose was incubated with arginine and pyridoxal phosphate in the cell-free extract without added ATP, protein was removed by ultrafiltration, and

the filtrate passed through Dowex 50X2 (NH_4^+) resin. Under these conditions, about 89% of the total radioactivity was eluted with water. Subsequent elution with 1N NH_4OH yielded an amino acid fraction that carried 1.9% of the total radioactivity. TLC analysis of this fraction indicated that the radioactivity was not due to the presence of ribose or sinefungin, and isotopic trapping showed that only 0.012% of the radioactivity in the amino acid fraction was due to the presence of sinefungin. When arginine was omitted from the incubation mixture, no radioactivity eluted in the ammonia wash. From these observations, it appears that arginine and the labeled ribose are converted into a labeled amino acid derivative ("X") by the cell-free extract. Similarly, incubation of [U-^{14}C]-L-arginine, D-ribose, and pyridoxal phosphate in the extract without added ATP followed by chromatography on an Affi-Gel 601 boronate column[15] yielded 89% of the total radioactivity in the wash and 3.9% of the radioactivity in the *cis* diol fraction. Chromatography of the diol fraction did not reveal the presence of labeled sinefungin or arginine, and no radioactivity appeared in the diol fraction when ribose was omitted from the incubation. When radioactive "X" isolated from either the ion-exchange column or the boronate column was incubated with adenine in the cell-free extract, radioactive sinefungin was produced (0.33% incorporation of "X" derived from labeled arginine, 0.55% incorporation of "X" derived from labeled ribose). These observations indicate that the radioactive species "X" could be an intermediate in sinefungin biosynthesis.[16]

In summary, the results obtained by administration of doubly-labeled forms of ATP and adenosine to cell-free extracts of *S. griseolus* demonstrate that these compounds are catabolized in the extracts to give fragments that are then incorporated into sinefungin. Therefore, the suggestion[5] that ATP is a specific precursor of sinefungin cannot be substantiated at this time. The fact that [5'(*RS*)-5'-^{3}H, 1'-^{14}C]adenosine is incorporated into sinefungin without tritium loss rules out the intermediacy of A9145C in sinefungin biosynthesis. Incubation of arginine, ribose, and pyridoxal phosphate with the cell-free extract leads to the formation of a putative intermediate that contains both amino acid and *cis* diol functionality. This putative intermediate appears to be converted into sinefungin when incubated with adenine in the cell-free extracts. Nevertheless, the number of enzymatic reactions catalyzed by the cell-free extracts make it likely that a clear picture of sinefungin biosynthesis will only be obtained after purification of the enzyme or enzymes that are responsible for the biosynthesis of this interesting antibiotic.

Experimental Section

General. ^{1}H spectra were recorded on either a JEOL FX-90Q (90 MHz) or a Bruker AM-250 (250 MHz) spectrometer. All ^{1}H chemical shifts are given in parts per million downfield from tetramethylsilane. Mass spectra were run on Finnegan 3300 and CEC 111021-110B mass spectrometers. Melting points were measured on a Fisher-Johns apparatus and are uncorrected. Analytical thin-layer chromatography was performed with precoated Merck silica gel type 60 F-254 glass plates (0.25 mm layers) and with Merck precoated cellulose F glass plates (0.1mm layers). Preparative thin-layer chromatography was performed with precoated Whatman or Aldrich cellulose glass plates lacking in a fluorescent indicator (0.25 mm layers). Column chromatography was carried out using EM silica gel (200-400 mesh). HPLC analyses was carried out using a 4.6 x 150 mm Whatman Partisphere C_{18} reverse-phase column. A Spectra-Physics SP8700 solvent delivery system was used in conjunction with an Isco V4 UV monitor. Radioactivity measurements were accomplished using a Beckmann LS 3801 liquid scintillation counter with automatic quench correction. Aquasol 2 from New England Nuclear was used as the scintillation cocktail. The radiochemical purity of radioactive precursors was evaluated using a Berthold LB 2832 TLC Linear Analyzer interfaced with an Apple IIe microcomputer. Fermentations were

conducted in a New Brunswick Scientific G-25 rotary shaker, and cell-free extracts were prepared by sonication with a Branson Cell Disrupter 200. Protein concentrations were determined by the method of Bradford[17] using bovine serum albumin as a standard.

Materials. Sinefungin was obtained as a gift from Eli Lilly Corp., courtesy of Dr. Robert Hamill. Other chemicals were obtained from Aldrich Chemical Co. and Sigma Chemical Co. Affi-gel 601 was purchased from Bio-Rad Laboratories, while radioactive precursors were obtained from Amersham/Searle Corp. and American Radiolabeled Chemicals, Inc. Ultrafree-MC filters (10,000 Dalton cutoff) were obtained from Millipore Corp. Acetone was dried over 4 A molecular sieves; methanol was dried by distillation from magnesium turnings; acetonitrile was distilled from P_2O_5 and stored over 4A molecular sieves; pyridine was dried by distillation from KOH pellets.

Organism, Fermentation, and Preparation of Cell-free Extracts. *S. griseolus* was obtained from Eli Lilly Corp. The organism was maintained on slants prepared from the inoculum medium of Berry and Abbott[18] containing 15% agar. The organism was preserved either by lyophilization or by the use of soil cultures. For the preparation of cell-free extracts, 100 mL of sterile inoculum medium[18] in a 500 mL Erlenmeyer flask was inoculated with a loopful of soil culture, and the medium incubated at 30 °C and 250 rpm for 72 h. At the end of this time, the mycelium was harvested by centrifugation at 1,000 x g for 10 min and then washed 3x with 0.05 M phosphate buffer (pH = 7.0) containing 0.1% 2-mercaptoethanol, 1mM PMSF, and leupeptin (0.7 mg/l). The washed cells were suspended in 20 mL of 0.05 M phosphate buffer containing 10% glycerol, 0.1% 2-mercaptoethanol, 1 mM PMSF, and leupeptin (0.7 mg/l). The cells were disrupted by sonication for 5 min at output 6, with a 90% duty cycle, and the insoluble material was removed by centrifugation for 1 h at 100,000 x g. Nucleic acids were precipitated with streptomycin sulfate (final concentration of 2.5%) and removed by centrifugation at 5,000 x g for 10 min. Solid ammonium sulfate was added over 10 min to 40% of saturation. After an additional 20 min stirring, the precipitated protein was removed by centrifugation at 10,000 x g for 15 min. The supernatant was then brought to 70% saturation by addition of solid ammonium sulfate over 5 min. The mixture was stirred for an additional 30 min and the precipitated protein was collected by centrifugation at 10,000 x g for 15 min. The recovered protein pellet was dissolved in 2 mL of phosphate buffer identical in composition to that used for sonication, and the solution was dialyzed overnight against the same buffer.

Incubation Studies. Incubations were carried out by modification of a published procedure.[5] The incubation mixture contained in 500 uL about 2.6 mg of protein from the protein pellet obtained as described above, unlabeled precursors at a concentration of 4 mM, 0.1 mM pyridoxal phosphate, 0.1 mM $MgCl_2$, and the radioactive precursor(s). Incubations were carried out for 80 min at 37 °C, at the end of which time the solution was passed through a Millipore Ultrafree-MC filter (10,000 Dalton cutoff) to remove protein. HPLC analysis of the filtrate was carried out in the manner previously reported.[5] Isolation of the radioactive sinefungin was accomplished by evaporation of the filtrate to dryness in vacuo, addition of unlabeled sinefungin (20 mg) as carrier, and purification of the sinefungin by preparative TLC (cellulose, n-BuOH: HOAc: H_20, 2.4: 0.7: 1.0), R_f = 0.25. Additional purification of the sinefungin was accomplished by conversion to 2',3'-O-isopropylidenesinefungin and the corrresponding lactam as described below.

Preparation of 2'3'-O-Isopropylidenesinefungin (5). The procedure is a modification of that described by Hampton.[19] A solution of sinefungin (20 mg, 0.052 mmol), bis(4-nitrophenyl)phosphate (57 mg, 0.17 mmol), and 2,2-dimethoxypropane (0.2 mL, 1.6 mmol) in dry acetone was stirred at room temperature for

48 h. The reaction was quenched with tri-n-butylamine (100 μL) and the volatile materials removed in vacuo. The yellow-orange residue was stirred in benzene (2 mL) for 2 h, and the insoluble materials recovered by filtration. The solid was dissolved in a minimum of water and the solution applied to a small column of Dowex 50X2 resin (3-4 mL, 100-200 mesh, NH_4^+ form). The column was washed with water and the 2',3'-O-isopropylidenesinefungin eluted with excess 2N NH_4OH. The basic eluant was taken to dryness in vacuo, and the residue purified by preparative TLC (cellulose, n-BuOH: HOAc: H_2O, 2.5: 0.6: 1.0 v/v), R_f = 0.45. The yield was 14.5 mg (66%) of a colorless gum; ^{13}C NMR (D_2O, 75.5 MHz): δ 177.1 (C-10'), 154.8 (C-6), 152.3 (C-2), 148.0 (C-4), 139.7 (C-8), 118.3 (C-5), 114.9 (C-13'), 88.3 (C-1'), 80.3 (C-4'), 73.4 (C-2', C-3'), 55.0 (C-9'), 48.4 (C-6'), 36.7 (C-5'), 30.1 (C-8'), 28.2 (C-7'), 26.6 (C-11'), 25.0 (C-12'); HRMS: (M-18): Expected for $C_{18}H_{25}N_7O_4$: 403.19676. Found, 403.19741.

Preparation of 2',3'-O-Isopropylidenesinefungin Lactam (6). A solution of 2',3'-O-isopropylidenesinefungin (14 mg, 0.043 mmol) in dry methanol (5 mL) was heated to reflux for 72 h. After cooling, the solvent was removed in vacuo and the residue purified by preparative TLC (cellulose, n-BuOH: HOAc: H_2O, 2.4: 0.2: 1.0 v/v), R_f = 0.46. The yield was 10 mg (74%) of a colorless gum; ^{13}C NMR (D_2O, 75.5 MHz): δ 173.8 (C-10'), 156.1 (C-6), 153.2 (C-2), 149.0 (C-4), 140.0 (C-8), 119.3 (C-5), 114.9 (C-13'), 88.1 (C-1'), 81.1 (C-4'), 73.9 (C-2', C-3'), 50.7 (C-9'), 48.9 (C-6'), 40.1 (C-5'), 26.6 (C-11'), 25.8 (C-8'), 25.0 (C-12'); HRMS: M^+ : Expected for $C_{18}H_{25}N_7O_4$: 403.19676. Found, 403.19557.

Synthesis of [1'-^{14}C]Adenosine

[1-^{14}C]-1-O-Methyl-2,3,5-tri-O-acetyl-D-ribofuranose.[12] Sulfuric acid (7.8 uL) was added with stirring to a solution of [1-^{14}C]-D-ribose (15 mg, 0.10 mmol, 48 μCi) in dry methanol (1.0 mL) and the mixture stirred at ice-bath temperature for 18 h. The solution was then neutralized with dry pyridine (48 uL) and then taken to dryness in vacuo. The residue was redissolved in dry pyridine (0.24 mL) and distilled acetic anhydride (95 uL, 0.33 mmol) added with stirring and cooling. The ice bath was removed and the reaction mixture was allowed to stand at room temperature for two days. The mixture was then poured into ice-water and the aqueous solution was extracted repeatedly with chloroform. The combined chloroform extracts were washed with water, dried (Na_2SO_4), and the solvent removed in vacuo at room temperature to yield [1-^{14}C]-1-O-methyl-2,3,5-tri-O-acetyl-D-ribofuranose as a colorless syrup, 29 mg (99%); the ^1H NMR spectrum (90 MHz, $CDCl_3$) indicated that the product was about a 5:1 mixture of C-1 epimers: δ 2.1 (overlapping singlets, 3 x 3 H, CH_3CO), 3.32 (ca. 2.4 H, OMe, s), 3.48 (ca. 0.6 H, OMe, s), 4.2 (3 H, m), 5.0 (3 H, m).

[1-^{14}C]-1,2,3,5-Tetra-O-acetyl-D-ribofuranose (7).[12] [1-^{14}C]-1-O-Methyl-2,3,5-tri-O-acetyl-D-ribofuranose (29 mg, 0.1 mmol) was dissolved in acetic acid (0.30 mL), distilled acetic anhydride was added (71 uL). The mixture was stirred in an ice-bath and treated with sulfuric acid (7.6 uL). The ice-bath was removed and the yellow solution stirred at room temperature for 12 h. Ice was then added and the mixture extracted repeatedly with chloroform. The combined organic extracts were washed with water and with sat. aq. sodium bicarbonate. The dried (Na_2SO_4) organic phase was evaporated in vacuo to yield [1-^{14}C]-1,2,3,5-tetra-O-acetyl-D-ribofuranose as a colorless semi-solid, 29.6 mg (91%); sp. act. = 431 μCi/mmol; TLC (SiO_2): n-hexane: EtOAc, 6: 4, R_f = 0.30; ^1H NMR (250 MHz, $CDCl_3$): δ 2.0 (overlapping singlets, 4 x 3 H), 3.8 - 4.4 (m, 3 H), 5.0 - 6.0 (m, 2 H), 6.0 -6.2 (m, 1 H).

[1'-^{14}C]2',3',5'-Tri-O-acetyladenosine (8).[14] [1-^{14}C]-1,2,3,5-Tetra-O-acetyl-D-ribose (32.5 mg, 0.10 mmol) and adenine (14.3 mg, 0.11 mmol) were suspended in dry acetonitrile (1.6 mL) and stannic chloride (24 uL, 0.20 mmol) added with stirring. The reaction was stirred overnight at room temperature. The reaction mixture was then concentrated in vacuo and a solution of sodium bicarbonate added (65 mg, 0.5 mL H_2O). The solvents were then removed in vacuo and the residue extracted repeatedly with hot chloroform. The combined chloroform extracts were dried (Na_2SO_4) and evaporated in vacuo to give a residue that was purified by column chromatography (SiO_2, $CHCl_3$: CH_3OH, 9.5: 0.5, v/v). [1'-^{14}C]2',3',5'-Tri-O-acetyladenosine was obtained as a colorless oil , 33.6 mg (80%), sp. act. = 369 μCi/mmol; TLC (SiO_2, $CHCl_3$: CH_3OH, 9.5: 0.5, R_f = 0.37; ^{1}H NMR (90 MHz, $CDCl_3$): δ 2.0 (overlapping singlets, 3 x 3 H), 4.2 (m, 1 H), 4.4 (m, 2 H), 5.0 - 5.4 (m, 1 H), 5.6 (bs, 2 H, NH_2), 5.85 (t, 1 H), 6.10 (d, 1 H), 7.85 (s, 1 H), 8.30 (s, 1 H).

[1'-^{14}C]Adenosine (9). [1'-^{14}C]-2',3',5'-Tri-O-acetyladenosine (33.6 mg, 0.08 mmol) was dissolved in a cold solution of ammonia saturated methanol (5 mL) contained in a thick-walled glass reaction tube (Ace Glass). The tube was sealed with a Teflon stopper and then heated at 50 °C for 10 h. At the end of this time, the tube was allowed to cool to room temperature, the stopper was removed, and the solution taken to dryness in vacuo. The labeled adenosine was isolated from the residue by preparative TLC (cellulose, n-BuOH: HOAc: H_2O, 4: 1: 1.5 v/v). [1'-^{14}C]Adenosine (9) was obtained as a white solid, 10.3 mg (53%), sp. act. = 427 μCi/mmol; the compound exhibited a single spot on TLC with an R_f identical to that of the authentic compound (cellulose, n-BuOH: HOAc: H_2O, 4: 2: 2), R_f = 0.57.

Isolation of the Putative Intermediate "X."

A. By Ion-exchange Chromatography. An incubation was carried out under standard conditions in the presence of [1-^{14}C]-D-ribose (0.3 mg, 10 μCi) and in the absence of added ATP. At the end of 80 min, protein was removed using a Millipore Ultrafree filter and the filtrate taken to dryness in vacuo at room temperature (freeze-dried?). The residue was dissolved in water (2 mL) and applied to a small column of Dowex 50x2 (100-200 mesh,10 mL, NH_4^+ form). The column was washed with water (30 mL) and the amino acids were then eluted with 1 N NH_4OH (25 mL). The amount of radioactivity in the aqueous wash and in the amino acid eluant was quantitated by liquid scintillation counting. The wash contained 8.9 μCi, while the eluant contained 1.9 μCi. Evaporation of the NH_4OH eluant in vacuo at room temperature gave a residue that was examined by TLC (cellulose, n-BuOH: HOAc; H_2O, 2.4: 0.7: 1.0). No sinefungin appeared to be present. In order to access more accurately the amount of radiolabeled sinefungin that might be produced under these conditions, the experiment was repeated. In this case, 10 mg of sinefungin was added to the ammonium hydroxide eluant. The sinefungin was then reisolated and purified by conversion to the lactam **6**. The lactam carried only 0.012% of the total radioactivity present in the amino acid containing eluant.

B. By Boronate Chromatography. An incubation was carried out under standard conditions in the presence of [U-^{14}C]-L-arginine (0.35 mg, 6.8 μCi) and in the absence of added ATP. At the end of 80 min, protein was removed by filtration and the filtrate evaporated in vacuo. The residue was dissolved buffer A (1 mL, 0.05M N-methylmorpholinium acetate, 0.05 M $Mg(OAc)_2$, 0.025 M KOAc, pH 9.1) and applied to a column of Affi-Gel 601(1.2 cm x 12 cm) equilibrated with buffer A. The column was washed with 18 mL of buffer A, followed by 6 mL of 0.02 M N-methylmorpholinium acetate, pH 9.1. The *cis* diol containing products were eluted with 44 mL of 0.25 M NH_4OAc, pH 5.0. The radioactivity in the buffer A wash and ammonium acetate

eluant was quantitated by liquid scintillation counting. The buffer A wash contained 6.0 μCi, while the ammonium acetate eluant contained 0.25 μCi.

Conversion of Putative Intermediate "X" into Sinefungin

A. "X" Derived from Labeled Ribose. A total of 0.19 μCi of the radioactive fraction eluted from the Dowex 50x2 column with 1 N NH_4OH was incubated under standard conditions in 500 uL of cell-free extract that was 4 mM in adenine. At the end of 80 min, the extract was deproteinized, radioinactive sinefungin was added as carrier, and the sinefungin was then reisolated and converted into the lactam **6** in the usual manner. The incorporation of "X" into **6** amounted to 0.55%.

B. "X" Derived from Labeled Arginine. A total of 0.23 μCi of the radioactive fraction eluted from the Affi-Gel 601 column with 0.25 M NH_4OAc was incubated under standard conditions in 500 uL of cell-free extract that was 4 mM in adenine. At the end of 80 min, the protein was removed by ultrafiltration, radioinactive sinefungin was added, and the sinefungin was then reisolated and purified by conversion to the lactam **6**. The incorporation of "X" into **6** was 0.33%.

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