BIOSYNTHETIC STUDIES OF ARIDICIN ANTIBIOTICS†

I. LABELING PATTERNS AND OVERALL PATHWAYS

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Biosynthetic feeding experiments with ^{14}C and ^{13}C-labeled precursors in *Kibdelosporangium aridum* have established the biosynthetic origins of the heptapeptide aglycone of the aridicin antibiotics, and the tentative sequence of the later stage biosynthetic transformations. The aglycone moiety has been found to be derived from tyrosine, sodium acetate and L-methionine. It is suggested that the preformed aglycone is first mannosylated and then followed by the attachment of the glycolipid. The sugar oxidation to the glucuronic acid level was found to take place as a terminal step of the biosynthesis.

Aridicins are a series of complex glycopeptide antibiotics related to the vancomycin family.[1] They are produced by a new genus of *Actinomycetales*, *Kibdelosporangium aridum* (ATCC 39323).[2,3] Three major components of the antibiotics, *i.e.*, aridicins A, B and C have been isolated and their structures recently determined in our laboratories.[4,5] These antibiotic components possess an identical aglycone structure and differ only in the nature of the *N*-acyl side chain of the 2-amino-2-deoxy-glucopyranuronic acid moiety. From a related species, *K. aridum* subsp. *largum*, subsp. nov. (ATCC 39922), an analogs series of antibiotics have also been isolated, in which a glucosaminyl residue has been found in lieu of the corresponding glucuronic acid unit of aridicins (Fig. 1).

The glycopeptide antibiotics of the vancomycin class have been a subject of current interest because of the increasing importance of vancomycin for treatment of methicillin-resistant staphylococcal infections and pseudomembraneous colitis, in addition to their potential as livestock growth promotants. The glycopeptide antibiotics are believed to exert their biological activity by interfering with cell-wall biosynthesis presumably through a strong binding to the cell-wall precursors terminating with L-Lys-D-Ala-D-Ala.[1] In this paper, we report results of our biosynthetic studies of the aridicin antibiotics which establish the biosynthetic origins of the heptapeptide aglycone and the sequence of the subsequent transformations.

Experimental

Organisms

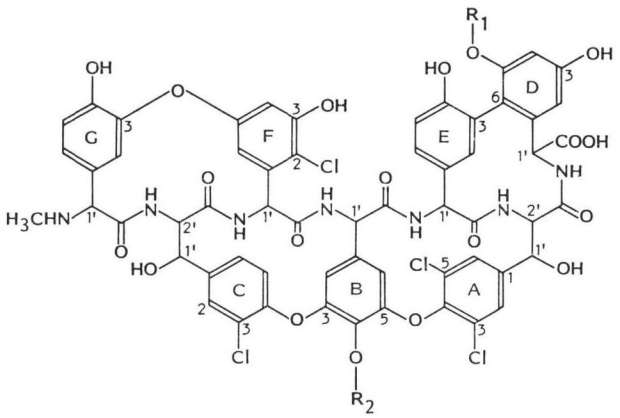
K. aridum (ATCC 39323; SK&F-AAD-216) and a related species *K. aridum* subsp. *largum*, subsp. nov. (ATCC 39922; SK&F-AAD-609) have been used as producers of aridicins and the 609 antibiotic complexes.[2] *Actinoplanes teichomyceticus* nov. sp. (ATCC 31121) was used in the deacylation experiments.[6]

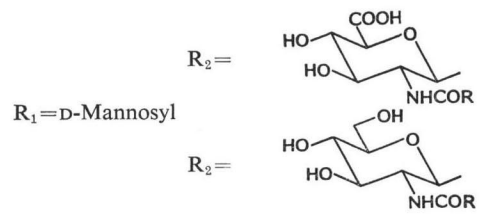
Culture Conditions

The seed culture was grown from an agar slant in a medium (13H) composed of the following

† The name of aridicin antibiotics was recently changed to ardacin.

Fig. 1. Structures of aridicin antibiotics.





$R=C_9H_{19}$ (Aridicin A)
$R=C_{10}H_{21}$ (Aridicin B)
$R=C_{11}H_{23}$ (Aridicin C)

$R=C_9H_{19}$ (AAD-609 A)
$R=C_{10}H_{21}$ (AAD-609 B)
$R=C_{11}H_{23}$ (AAD-609 C)

ingredients; distilled water 1 liter, starch 15 g, sucrose 5 g, dextrose 5 g, soy peptone 7.5 g, corn steep liquor 5 g, K_2HPO_4 1.5 g, NaCl 0.5 g, $CaCO_3$ 1.5 g, mineral supplement 5 ml of $ZnSO_4 \cdot 7H_2O$ 2.8 g/liter, ammonium ferric citrate 2.7 g/liter, $CuSO_4 \cdot 5H_2O$ 0.125 g/liter, $MnSO_4 \cdot H_2O$ 1 g/liter, $CoCl_2 \cdot 6H_2O$ 0.1 g/liter, $Na_2B_4O_7 \cdot 10H_2O$ 0.1 g/liter, $Na_2MoO_4 \cdot 2H_2O$ 0.05 g/liter. The pH of the seed medium was adjusted to 7.0, and the seed culture was usually incubated at 28°C for 4 days on a shaker at 250 rpm and 5-cm throw.

The production medium of aridicins (V-2) contained the following ingredients; distilled water 1 liter, soybean meal 15 g, beet molasses 10 g, Estransan-4 10 g, glucose or glycerol 10 g and NaCl 0.3 g. The pH was adjusted to 7.0 with NaOH. Typically, 50 ml of the production medium in a 250-ml shake flask was inoculated with 5~10 ml of the seed culture and incubated for 5~7 days at 28°C and 250 rpm. In the feeding experiments, most of the precursors were filtered through a disposable, sterile Millipore filter (0.45 μm) before addition to the sterile cultures at 0~72 hours after inoculation. Some precursors with low water solubility were added as solids.

The composition of the chemically defined medium (MBSM-2) was as follows; distilled water 890 ml, $MgCl_2 \cdot 7H_2O$ 0.25 g, $CaCl_2 \cdot H_2O$ 0.01 g, NaCl 1 g, $(NH_4)_2SO_4$ 0.1 g and glucose or glycerol 8.9 g. The seed culture was washed with distilled water three times before inoculation into this medium. The production of aridicins in this medium was usually 10~20% of that in the complex medium.

Isolation and Purification

The isolation and purification of aridicins were accomplished according to the published procedures,[3] with the following modifications. At the end of fermentation, the broth was centrifuged to remove the mycelia (9,000 rpm for 15 minutes) and the mycelia was washed with an equal volume of CH_3CN - H_2O (50: 50). The combined broth supernatant and the washing were worked up as described previously to yield the aridicin antibiotics.[3]

Table 1. Incorporation of ^{14}C-substrate into aridicins.

Precursor	Medium	Feeding-time (hours)	Incorporation (%)
L-[*U*-^{14}C]Tyrosine	V-2/glucose[a]	48	0.9
	V-2/glycerol[a]	48	8.0
	V-2/glycerol[b]	38	19.5
		43	29.1
		48	20.2
		53	15.9
		58	2.5
Sodium [1-^{14}C]acetate	V-2/glycerol[b]	24	0.1
		42	0.7
		48	0.8
		66	0.8
L-[*methyl*-^{14}C]Methionine	V-2/glycerol[b]	16	2.0
		24	2.7
		40	5.2

[a] In these experiments, the mycelial washing procedure was not used in the work-up.
[b] The work-up included the mycelial washing procedure.

Isotope-labeled Substrates

L-[*U*-^{14}C]Tyrosine (specific activity 450 mCi/mmol), sodium [1-^{14}C]acetate (specific activity 45~60 mCi/mmol), L-[*methyl*-^{14}C]methionine (specific activity 40~55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Sodium [1-^{13}C]acetate (99 atom %), sodium [2-^{13}C]acetate (99 atom %), DL-[3-^{13}C]-4-hydroxyphenylalanine (99 atom %), DL-[2-^{13}C]-4-hydroxyphenylalanine (99 atom %), L-[*methyl*-^{13}C]methionine (99 atom %) were purchased from Merck Sharp & Dohme Isotopes (St. Louis, MO). The ^{14}C-labeled hydrolysis products, [^{14}C]aglycone, [^{14}C]mannosylaglycone, and [^{14}C]pseudoaglycone-I were prepared from ^{14}C-labeled aridicin complex which was, in turn, obtained from fermentation of L-[*U*-^{14}C]tyrosine.

Analytical Methods

High performance liquid chromatography (HPLC) was performed on either a Spectra Physics 8100/4000 liquid chromatograph or an LDC Constametric HPLC system using 7~34% CH_3CN in 0.01 M KH_2PO_4 buffer (pH 3.2) and detection at 220 nm. A Beckman Ultrasphere ODS column (4.6×150 mm, at flow rate 1.5 ml/minute) and a Beckman Ultrasphere ODS semi-prep column (10×250 mm, at flow rate 6.0 ml/minute) were used for the analytical and preparative purposes, respectively.

Counting was performed on a Tracor Analytic Mark III-6882 Liquid Scintillation System using Beckman Ready-Solv HP/b counting cocktail. The ^{13}C NMR spectra were taken on a Bruker Instruments WM-360 spectrometer operating at 90.56 MHz at ambient temperature using a 5 mm $^{13}C/^1H$ probe. NMR samples were dissolved in CD_3OD - D_2O (20: 80, pH 8.8) and are given in the δ-scale.

Results and Discussion

The literature precedents on the biosynthesis of vancomycin,[7] ristocetin[8] and avoparcin[9] and the structural information on the aridicin antibiotics suggested that the β-hydroxytyrosine and *p*-hydroxyphenylglycine residues of the aridicin complex are most likely derived from tyrosine, whereas 3,5-dihydroxyphenylglycine may be of polyketide origin. Therefore, the initial feeding experiments were carried out with L-[*U*-^{14}C]tyrosine, sodium [1-^{14}C]acetate and L-[*methyl*-^{14}C]methionine in terms

Table 2. Incorporation of ^{13}C-substrate into aridicins.

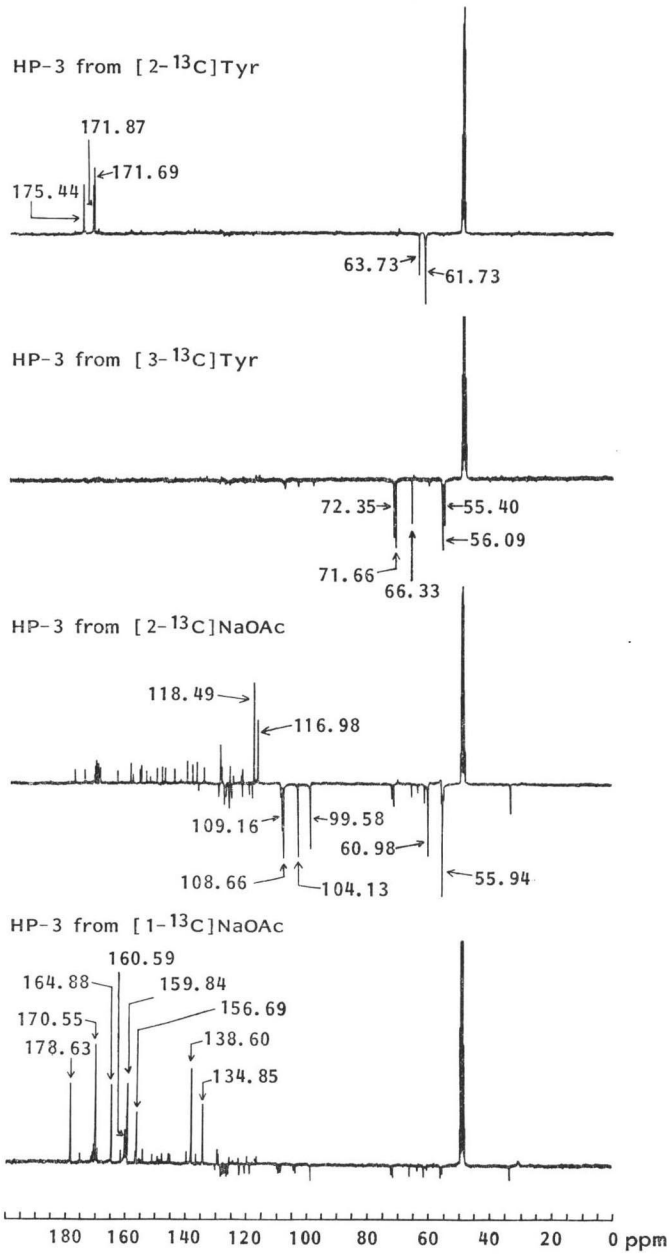
Precursor (amount)	Feeding-time (hours)	Enhanced peaks in product
DL-[3-^{13}C]Tyrosine (0.1 g/100 ml)	64	Aridicin aglycone: δ 72.35 (A1′), 71.66 (C1′), 66.33 (G1′), 56.09 (B1′), 55.40 (E1′)
DL-[2-^{13}C]Tyrosine (0.1 g/200 ml)	52	Aridicin aglycone: δ 175.44, 171.87 and 171.69 (carbonyl carbons in B, E and G), 63.73 (C2′), 61.73 (A2′)
Sodium [1-^{13}C]acetate (1 g/500 ml)	52	Aridicin aglycone: δ 178.63 and 170.55 (carbonyl carbons in D and F), 164.88, 160.59, 159.84 and 156.69 (D3, D5, F3, F5), 138.60 and 134.85 (D1, F1)
Sodium [2-^{13}C]acetate (1 g/500 ml)	52	Aridicin aglycone: δ 118.49 (D6), 116.98 (F2), 109.16 (D2), 108.66 (F4), 104.13 (F6), 99.58 (D4), 60.98 (D1′), 55.94 (F1′)
L-[*methyl*-^{13}C]Methionine	52	Aridicin A: δ 32.43 (*N*-CH_3)

of finding the optimum incorporation conditions. Results of these experiments are summarized in Table 1. The percent incorporation of the labeled precursors into the aridicin complex was found to be highly dependent on the carbon source added and the precursor feeding time. In the case of tyrosine, a relatively narrow feeding-time window exists for the optimum utilization of the precursor, and this feeding window could be nicely correlated to *ca.* 5~10 μg/ml production level of aridicins A, B and C. Sodium acetate unquestionably serves as a precursor, but the incorporation was invariably low. Several observations may be relevant to the poor utilization of the sodium acetate in the aridicin production. It was found that the aridicin production was substantially suppressed by the presence of the 2 mg/ml level of sodium acetate. A similar suppression effect was also observed in the presence of 0.5 mg/ml D-*p*-hydroxyphenylglycine. As the sodium [2-^{13}C]acetate feeding experiment has indicated (see later), sodium acetate is apparently an inferior source of acetyl-CoA in the *K. aridum* metabolism compared to methyl oleate (Estrasan-4) present in the production medium (V-2).

The labeling pattern of these precursors in the aridicin aglycone was investigated by means of ^{13}C NMR spectroscopy. The ^{13}C-labeled precursors were supplemented to the fermentation broth (V-2/glycerol medium) at the indicated time and the fermentation continued for an additional 3 to 5 days. The antibiotics were isolated as previously described.[3] The products, either fractionated by preparative HPLC into the individual components, or directly hydrolyzed to aridicin aglycone,[4] were analyzed by ^{13}C NMR. The results of the ^{13}C-precursor incorporation are shown in Table 2 and Fig. 2. The natural abundance ^{13}C NMR spectrum of aridicin aglycone in CD_3OD - D_2O (1 : 4) shows all 59 carbons; 7 carbonyl carbons, 25 aromatic carbons containing a substituent, 17 aromatic carbons bearing hydrogen, 9 methine carbons bearing oxygen or nitrogen, and the *N*-methyl carbon. Of these carbon resonances, the *N*-methyl carbon, methine carbon and the hydrogen-bearing aromatic carbon signals have been reasonably well assigned on the basis of chemical shift considerations and the results of a 2-dimensional $^{13}C/^{1}H$ COSY spectrum.[5]

The aridicin aglycone derived from DL-[3-^{13}C]tyrosine showed five enriched carbons assignable to A1′ (72.35 ppm), B1′ (56.09), C1′ (71.66), E1′ (55.40) and G1′ (66.33), while the DL-[2-^{13}C]tyrosine-derived aglycone displayed unambiguous signal enhancements at carbonyl carbons (175.44, 171.87 and 171.69 ppm) and two methine carbons; A2′ (61.73) and C2′ (63.73). In the case of DL-[2-^{13}C]-tyrosine-derived avoparcin, the reported enrichment was sufficiently low that incorporation at some carbonyl carbons that were expected to be derived from the labeled tyrosine could not be confidently

Fig. 2. Gated spin echo-edited ^{13}C NMR spectra of the biosynthetically enriched aridicin aglycones where the CH and CH_3 are negative, and the CH_2 and $C_{quat.}$ are positive.



claimed.[9] Although specific assignments of the carbonyl resonances were not made, the labeling patterns in the tyrosine-derived antibiotics are clearly consistent in terms of the number of the enhanced resonances with the biosynthetic scheme shown in Fig. 3.

From the sodium [1-^{13}C]acetate feeding experiment, aridicin aglycone was obtained in which enhancements were observed at two carbonyl carbons (178.63 and 170.55 ppm), four oxygen-bearing aromatic carbons (164.88, 160.59, 159.84 and 156.69) and two substituted aromatic carbons (138.60 and 134.85). On the other hand, the sodium [2-^{13}C]acetate feeding provided aridicin aglycone in

Fig. 3. Biosynthetic origins of aridicin aglycone.

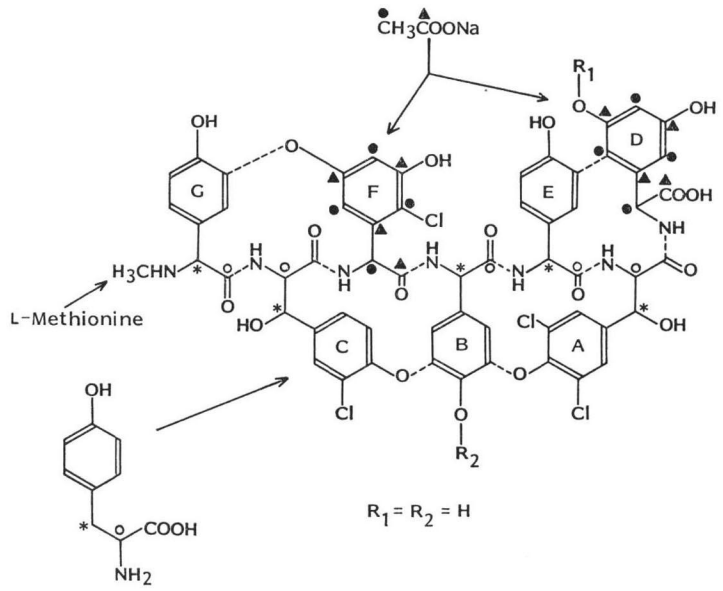


Table 3. Effect of potentially competing precursors on the L-[*U*-^{14}C]tyrosine incorporation into aridicins.*

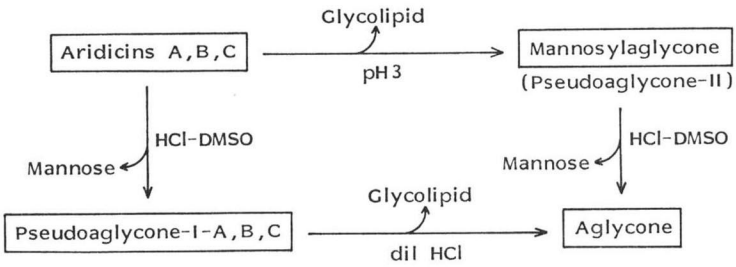
Potential precursor	Incorporation of L-[*U*-^{14}C]-tyrosine
None (control)	2.1
p-Hydroxyphenylpyruvate	2.1
p-Hydroxyphenylacetate	2.4
DL-*p*-Hydroxymandelic acid	1.4
p-Hydroxyphenylglyoxylate	0.4

* L-[*U*-^{14}C]Tyrosine (5 μCi each) and the potential precursor (1 mg each) were added to the fermentation broth (10 ml of V-2/glycerol) at 30 hours after inoculation.

which peak enhancements were noted at two substituted aromatic carbons (118.49 and 116.98 ppm), four hydrogen-bearing aromatic carbons; D2 (109.16), F4 (108.66), F6 (104.13) and D4 (99.58), and two methine carbons; D1′ (60.98) and F1′ (55.94). The corresponding aridicin A showed eight enhanced peaks at δ 120.68, 116.80, 110.52 (D2), 108.71 (F4), 103.43 (F6), 99.63 (D4), 60.31 (D1′) and 55.66 (F1′). The spectral comparison between the sodium [2-^{13}C]acetate-derived aglycone and the corresponding aridicin A strongly suggests that the shifted peaks at 120.68 and 118.49 may be assigned to the D6 carbon of aridicin A and aglycone, respectively. From the above results, it may be concluded that the 3,5-dihydroxyphenylglycine which constitutes rings D and F are derived from an acetate precursor as previously suggested.[7~9]

It is intriguing to note that the aridicin A samples derived from ^{13}C-labeled sodium acetate did not show any appreciable signal enhancement for the fatty acid side chain carbons. This observation plus the fact that the methyl oleate addition to the production medium significantly increases the antibiotic production, may indicate that in this microorganism, the fatty acid side chains are primarily derived from methyl oleate *via* the β-oxidative pathway, whereas sodium acetate serves as a precursor to the polyketide units; *i.e.*, there exists a substantial partition barrier between the fatty acid and the polyketide pathways. The feeding experiment with L-[*methyl*-^{13}C]methionine provided aridicin A in which the *N*-CH_3 group at δ 32.43 ppm was found to be the only enriched carbon, as anticipated.

Scheme 1. Hydrolysis products of aridicin antibiotics.



The natural occurrence of *p*-hydroxyphenylglycine (*p*-HPG) in secondary metabolites is relatively infrequent, the best known examples being nocardicin A and the glycopeptide antibiotics. Several possible pathways have been considered for the conversion of L-tyrosine to D-*p*-hydroxyphenylglycine in the biosynthesis of nocardicin A.[10,11] On the basis of the [2-^{13}C]tyrosine feeding experiment, the pathway involving carboxyl migration to the benzylic position followed by loss of the aminomethyl group can be readily excluded in the biosynthesis of aridicin. In order to gain some insight into this conversion, we carried out a series of isotope-competition experiments[12] with L-[*U*-^{14}C]tyrosine and potential intermediates between tyrosine and *p*-HPG, and the results are shown in Table 3. In the control, ^{14}C-labeled tyrosine alone was added to the fermentation broth of *K. aridum*, while various unlabeled potential precursors were supplemented together with the labeled tyrosine in the competition experiments. In spite of the imprecise nature of this kind of experiment, *p*-hydroxymandelic acid and *p*-hydroxyphenylglyoxylic acid appear to favorably compete with the labeled tyrosine. The finding seems to be in line with the pathway initially suggested by HOSODA *et al.*[11]

The β-hydroxylation in the conversion of tyrosine to (2*R*,3*R*)- and (2*S*,3*R*)-β-hydroxytyrosine of vancomycin was previously reported to occur with retention of configuration at C-3, irrespective of the C-2 stereochemistry.[7] It seems most likely that the glycopeptide antibiotic biosynthesis takes place in an environment in which the racemase(s) responsible for the α-epimerization of tyrosine and *p*-HPG are abundantly present.

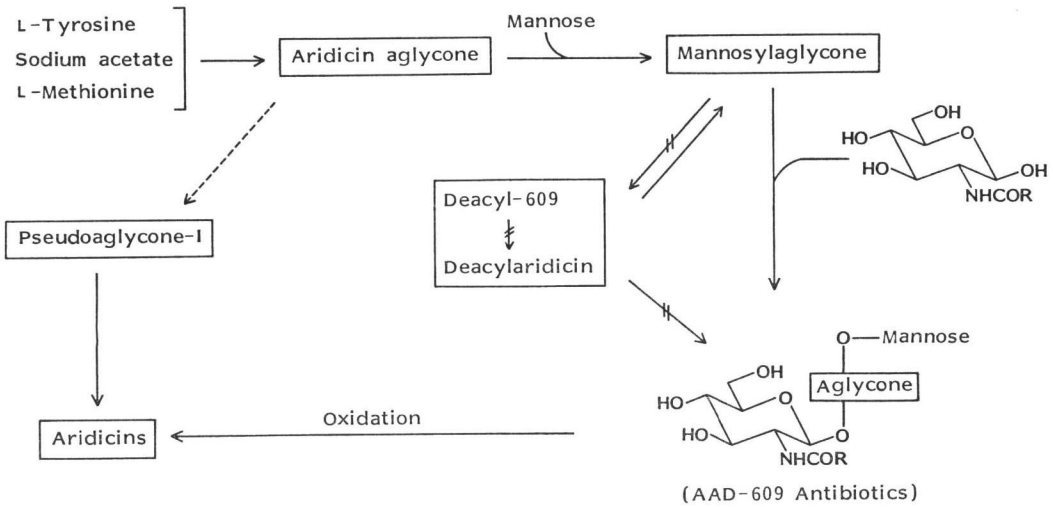
Next, we examined the biosynthetic sequence of glycosylation in the aridicin antibiotics. The previous study on the hydrolysis of aridicins made it possible to selectively prepare mannosylaglycone (pseudoaglycone-II), glycolipid-containing aglycone (pseudoaglycone-I) and the aglycone (Scheme 1).[4] The initial supplementation experiments in which the addition of unlabeled precursors were followed by HPLC based time-course studies have conclusively shown that the aglycone and the pseudoaglycone-I (A, B and C) were almost quantitatively mannosylated within 24 hours to mannosylaglycone and aridicin (A, B and C), respectively, when added to the fermentation broth of *K. aridum* 48 hours after inoculation. The half-reaction time for these mannosylations was estimated to be *ca.* 5 hours. The mannosylating activity was found to be present not only during the early production phase but also in the growth phase, albeit at a somewhat lower level. It is interesting to note that the mannosylation could also be seen in a culture of *K. aridum* in which the antibiotic production was largely suppressed by addition of 1 mg/ml phosphonomethylglycine (glyphosate; trade name Roundup), known to inhibit shikimate biosynthesis, thereby blocking aromatic amino acid production.[13]

Since the conversion of mannosylaglycone to aridicins could not be confidently demonstrated by the HPLC-based time-course study, radio-labeled precursor feeding experiments were carried out.

Table 4. Conversions of ^{14}C-labeled precursors.

Strain	Precursor	Feeding-time (hours)	Duration of fermentation	Product (Incorporation %)
AAD-216	[^{14}C]Aglycone	48	18 hours	Mannosylaglycone (74%)
AAD-216	[^{14}C]Aglycone	48	4 days	Aridicin complex (1.2%)
AAD-216	[^{14}C]Mannosylaglycone	48	4 days	Aridicin complex (6.0%)
AAD-609	[^{14}C]Aglycone	48	5 days	Aridicins *via* AAD-609 (2.0%)
AAD-609	[^{14}C]Mannosylaglycone	48	5 days	Aridicins *via* AAD-609 (1.75%)
AAD-609	[^{14}C]Deacylaridicin[14])	48	5 days	Mannosylaglycone (37.5%) and aridicins (0.45%)

Scheme 2. Overall biosynthetic pathways of aridicin antibiotics.



The ^{14}C-labeled aridicin complex, obtained from L-[*U*-^{14}C]tyrosine, was hydrolyzed to aglycone and mannosylaglycone, and these precursors were purified by preparative HPLC. In a series of experiments, the radio-labeled precursors were supplemented to *K. aridum* in V-2/glycerol. At the end of fermentations, the products were isolated, purified by preparative HPLC and counted. The conversions of the ^{14}C-labeled aglycone and mannosylaglycone are summarized in Table 4. Mannosylaglycone was clearly converted to aridicins with a reasonable efficiency. The biotransformation of aglycone to aridicins also occurs at a somewhat lower efficiency. This low conversion of aglycone to the aridicin complex is surprising, especially in view of the fact that the mannosylation of aglycone is very efficient. It is also noteworthy that the efficiency for the conversion of mannosylaglycone to aridicins is much lower than that of mannosylation. One possible reason may be that while the mannosylation takes place in the outer surface of the cytoplasmic membrane, the site of the glycolipid attachment lies deeper inside the cytoplasmic membrane. In fact, a protoplast preparation of *K. aridum* has been found to have mannosylating activity.[14]) Although efficiently converted to aridicins, the possible role of

pseudoaglycone-I in the biosynthesis of the aridicin is not yet clear. It is speculated that the mannosylation of pseudoaglycone-I may not be a part of the major biosynthetic pathway of aridicins, especially since the same biotransformation could be seen in the fermentation of other glycopeptide antibiotic producing strains such as *A. teichomyceticus*.[14]

Aridicins are the most highly oxidized among the glycopeptide antibiotics reported to date. The stage of oxidation of the glucose moiety to the glucuronate has been examined. When the AAD-609 antibiotics (A, B and C separately) were supplemented to the *K. aridum* fermentation broth 48 hours after inoculation, the HPLC-based time-course studies convincingly showed that these antibiotics were cleanly converted to the corresponding aridicins within 24 hours. This observation is also corroborated by the feeding experiments shown in Table 4. The labeled precursors were fed to the fermentation broths of AAD-609 in E1 medium, a condition in which the AAD-609 antibiotics appeared in the early phase and then subsequently replaced by aridicins. The aglycone and mannosylaglycone were found to be converted to aridicins *via* the AAD-609 antibiotics in 2.0% and 1.75% efficiencies, respectively. This finding suggests that the oxidation at C-6 in the *N*-acylglucosamine moiety may take place at the last stage of the biosynthetic pathway. The possible intermediacy of deacylaridicin, obtained from the over-fermentation of *A. teichomyceticus* with supplemented aridicins,[14] was also examined. When the ^{14}C-labeled deacylaridicin was fed to the AAD-609 fermentation broth, mannosylaglycone and aridicins were obtained in 37.5% and 0.45% radiochemical yield, respectively. In the HPLC-based time-course study, both deacylaridicin and deacyl-609 antibiotic were found to be efficiently converted to mannosylaglycone. These observations suggest that the AAD-609 broth possesses an aminoglucosidase activity and that the low (0.45%) conversion of deacylaridicin to aridicin is most likely due to the initial hydrolysis to mannosylaglycone followed by its subsequent utilization in the aridicin biosynthesis. Based on the above results, the later stage pathways in the aridicin biosynthesis may be constructed as shown in Scheme 2.

The possibility of manipulating the degree and the nature of halogen present in the aridicin antibiotics was also investigated. However, when extensively washed seed cells of *K. aridum* were used to inoculate a chemically defined medium (MBSM-2) completely devoid of chloride ion sources, the antibiotic production level was dramatically decreased. Furthermore, an extensive mass spectral analysis of the antibiotics thus produced failed to show any presence of dechlorinated aridicins in the antibiotic complex, although the chemically produced dechloroaridicins and their derivatives clearly showed the characteristic molecular ion peaks (CHUNG, S. K.: unpublished results). When the chemically defined medium in which the chloride salts were completely replaced by the corresponding bromide salts was inoculated with the washed seed cells, neither antibiotic production, nor healthy cell growth was observed. Therefore, the presence of the chloride ion seems to be required either for the normal cell metabolism or for antibiotic production.

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