BIOSYNTHETIC STUDIES OF ARIDICIN ANTIBIOTICS†

II. MICROBIAL TRANSFORMATIONS AND GLYCOSYLATIONS BY PROTOPLASTS

S. K. CHUNG*, Y. K. OH, P. TAYLOR, R. GERBER and L. J. NISBET

Smith Kline & French Laboratories
Philadelphia, PA 19101, U.S.A.

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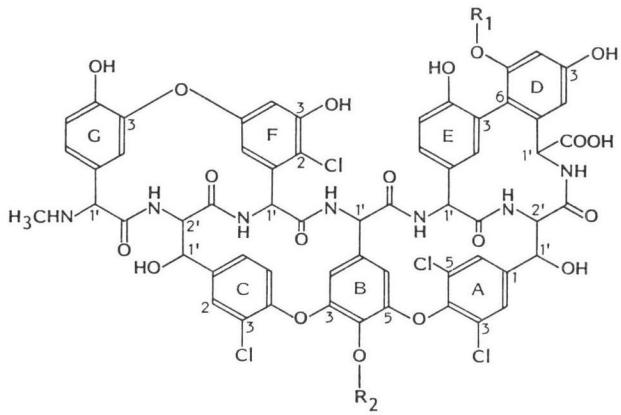
In connection with the biosynthetic studies of the aridicin antibiotics in *Kibdelosporangium aridum*, various microorganisms, known to produce related glycopeptide antibiotics, have been examined for their glycosylating activity. A number of strains were found to mannosylate the aridicin aglycone, while *Actinoplanes teichomyceticus* was found to have deacylating activity as well. A protoplast system of *K. aridum* was developed and was found to possess novel glycosylating activity in addition to the mannosylating activity which was also present in the whole cells. Effects on the glycosylating activity by various membrane solubilizing agents have been discussed.

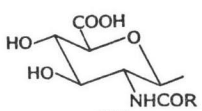
Aridicins are a series of complex glycopeptide antibiotics classified to the vancomycin-ristocetin family,[1] and produced by a new genus of *Actinomycetales*, *Kibdelosporangium aridum* (ATCC 39323).[2,3] Three major components of these antibiotics, aridicins A, B and C, were isolated and their structures were elucidated (Fig. 1).[4,5] The recent biosynthetic studies have shown that the origins of the basic building blocks of the heptapeptide aglycone moiety of the antibiotics are tyrosine, L-methionine

Fig. 1. Structures of aridicin antibiotics.



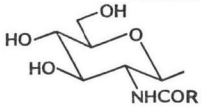
R_1=D-Mannosyl

R_2=



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

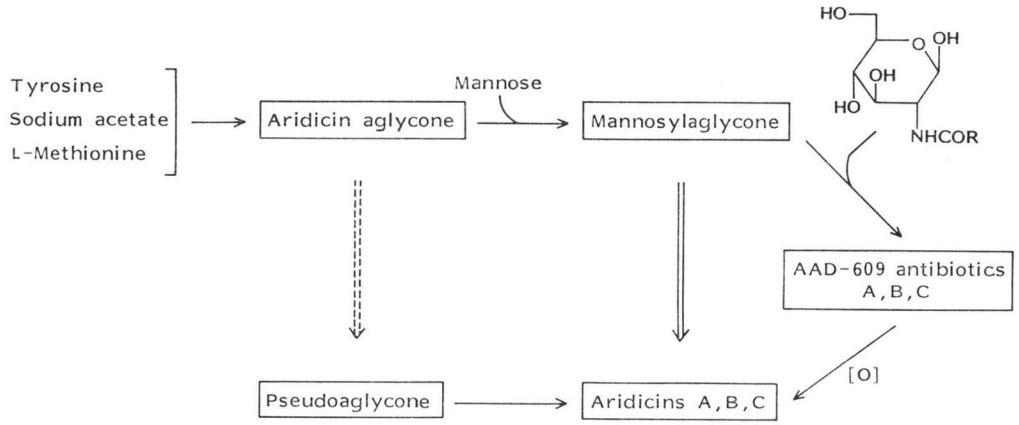
R_2=



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

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

Scheme 1. Proposed overall biosynthetic pathways of the aridicin antibiotics.



and sodium acetate derived polyketide.[6] The biosynthetic sequence subsequent to the aglycone formation was also investigated by feeding unlabeled and ^{14}C-labeled aglycone and pseudoaglycones, obtained from the hydrolysis of the parent antibiotics, to the fermentation broth of *K. aridum*. The suggested overall pathways for the biosynthesis of aridicins is shown in Scheme 1. In this report, we describe our studies on the biotransformations of aridicins and their derivatives by the whole cells of various glycopeptide producing microorganisms and also by a protoplast preparation of *K. aridum*.

Experimental

Culture Conditions

The sources of the microbial strains used are indicated in Table 1. Seed cultures were all grown from agar slants and incubated at 28°C for 4 days on a shaker at 250 rpm and 5-cm throw in the seed medium (13H), composition of which was previously reported.[6] The following production media were employed for the fermentation of various glycopeptide producing strains.

Medium V-2 was already described.[6]

Medium T (g/liter): Meat extract 4, peptone 4, yeast extract 1, NaCl 2.5, soybean meal 10, $CaCO_3$ 5 and glucose 5 in distilled water 1 liter at pH 7.0.

Medium E1 (g/liter): Yeast extract 1, $CaCO_3$ 1, $CoCl_2$ 0.001, soy peptone 10 and glucose 20 in distilled water 1 liter at pH 7.0.

Medium E5 (g/liter): Yeast extract 1, $CaCO_3$ 1, $CoCl_2$ 0.001, soy peptone 6.6 and soluble starch 20 in distilled water 1 liter at pH 7.0.

A representative fermentation was run as follows. A production medium (10 ml) in a shake flask (50-ml) was inoculated with 1~2 ml of seed culture and incubated for 3~8 days at 28°C and 250 rpm. In the precursor feeding experiments, the precursor was dissolved in water or other appropriate solvent mixture, and filtered through a disposable, sterile, pyrogen free filter (Schleicher and Schull, 0.45 μm) before the aseptic addition to the broth.

A typical deacylation with *Actinoplanes teichomyceticus* (ATCC 31121) was carried out as follows. The substrate, aridicins or AAD-609 antibiotics, was aseptically added to the actively fermenting broth in T medium 2~3 days after inoculation. Progress of deacylation was monitored by taking daily samples and analyzing them by HPLC. The retention times for aridicin A and deacylaridicin on an ODS reverse phase HPLC program of 7~34% CH_3CN in KH_2PO_4 buffer (pH 3.2) are 17.67 and 6.01 minutes, respectively. The deacylation was usually complete in 10 days.

Isolation and Purification

Isolation and purification of aridicins and related glycopeptides from the fermentation broths were

Table 1. Mannosylating activity of glycopeptide producing strains.

Strain	Glycopeptide produced	Medium	Mannose in native glycopeptide[a]	Mannosylation tested[b]	
				Aridicin aglycone to mannosyl-aglycone	Pseudoaglycone-I-A to aridicin A
K. aridum (ATCC 39323)	Aridicins	V-2	Yes	+	+
SK&F-AAD-609 (ATCC 39922)	609 A, B, C	E1	Yes	+	+
A. teichomyceticus (ATCC 31121)	Teicoplanin	T	Yes	+	+
A. missouriensis (ATCC 23342)	Actaplanin	E5	Yes	−	NT[c]
S. orientalis (NRRL 2450)	Vancomycin	E1	No	−	NT
S. orientalis[d] (NRRL 2452)	Vancomycin	E1	No	−	NT
S. candidus[d] (NRRL 3218)	Avoparcin	E1	Yes	−	NT
Actinomadura sp. (SK&F-AAJ-271)	Unknown	E1	Yes	+	NT
Nocardia sp. (SK&F-AAJ-516)	Ristocetin	E1	Yes	−	−
Unclassified (SK&F-AAJ-193)	Actinoidin	E1	Yes	+	+

[a] Yes and No denote the presence and absence of mannose in the native glycopeptide antibiotic respectively.

[b] Signs, + and −, respectively indicate success and failure of the indicated conversion with the whole cell fermentation of the strain in the indicated medium.

[c] NT: Not tested.

[d] In these strains, the mannosidase activity (conversion of mannosylaglycone to aglycone) was noted.

done essentially according to the procedure previously described.[6]

Analytical Methods

High performance liquid chromatography (HPLC) was performed on either a Spectra Physics 8100/4000 liquid chromatography or a LDC Constametric HPLC system using 7~34% gradient of CH_3CN in 0.01 M KH_2PO_4 buffer (pH 3.2) and a spectrophotometric detector at 220 nm. The following columns were used: Beckman Ultrasphere ODS column (4.6×150 mm at flow rate of 1.5 ml/minute) and Beckman Ultrasphere ODS semi-prep column (10×250 mm at flow rate of 6.0 ml/minute) for the analytical and preparative purposes, respectively. The preparative HPLC of the radio-labeled compounds was done on the LDC system using the ODS semi-prep column either isocratically or using step-gradients. For the HPLC analyses of broth samples the following Sep-pak procedure was employed. The C18 cartridge (Waters Associates, Milford, MA) was primed by successive washing with CH_3CN (4 ml), H_2O (4 ml) and 0.1 M KH_2PO_4 (pH 3.2, 4 ml). The sample broth (1~2 ml), after centrifugation, was passed through the cartridge and washed with 0.1 M KH_2PO_4 (2 ml). The retained material was eluted with 2 ml of 50% CH_3CN - 0.1 M KH_2PO_4 and used for the HPLC analysis.

The radioactivity counting was performed on a Tracor Analytic Mark III-6882 Liquid Scintillation System using Beckman Ready-Solv HP/b counting cocktail. The mass spectral analysis was carried out on a VG Analytical ZAB-IF mass spectrometer equipped with a high field magnet and operated in the fast atom bombardment (FAB) mode with glycerol as solvent matrix.

Material

Aridicin aglycone and pseudoaglycones were prepared from the parent antibiotics according to

the previously described procedures.[6] Preparation of the ^{14}C-labeled aridicins and their hydrolysis products have already been reported.[6] The lysozyme from chicken egg white (Grade 1, Sigma Chem. Co., St. Louis, MO) was used in the protoplast preparation. All other biochemicals and organic/inorganic chemicals were purchased from either Sigma Chem. Co. or Aldrich Chem. Co. (Milwaukee, WI), and used without further purification. Trypticase soy broth (TSB) was obtained from BBL & Falcon Products (Cockeysville, MD).

Protoplast Preparation (a Representative Procedure)

Mycelial cells of *K. aridum* (ATCC 39323) from 4 fresh plants were macerated by homogenation, inoculated into TSB (4×50 ml), and incubated for 24 hours on a shaker at 28°C and 250 rpm. Cells were collected by centrifugation, reinoculated into TSB containing 1.5% glycine (12×50 ml), and incubated for another 24 hours under the same conditions as above. The harvested cells were washed with P-solution,[7] and incubated in P-solution containing lysozyme (5 mg/ml) for 30 minutes at 37°C in a water bath. At the end of the incubation, the crude protoplast suspension was centrifuged for 5 minutes at *ca.* 1,000 rpm (IEC Clinical Centrifuge, angle rotor of 6×50 ml). The supernatant was separated and centrifuged again for 10 minutes at 3,750 rpm to provide protoplasts in pellet. The pellet was resuspended in P-solution to a concentration of $5\sim8\times10^8$ protoplasts/ml by using a hemacytometer (Bright-Line, A.O. Scientific).

In a typical glycosylation experiment, aridicin aglycone (1 mg in 1 ml of MeOH - H_2O, 7: 3) was added to the freshly prepared protoplast suspension (10 ml) and incubated in a reciprocating water bath (Precision Shaker Bath) at 30°C and 65 spm. At the end of the incubation, protoplasts were removed by centrifugation and the supernatant was worked up to yield the products.

Results and Discussion

The previous biosynthetic studies have shown that the preformed aglycone of aridicin serves as a viable precursor to the parent antibiotics. This and other bioconversion data have suggested an overall biosynthetic pathway in which glycosylations and other transformations such as *N*-acylation with fatty acids and oxidation of the sugar residue to glucuronate may follow the heptapeptide core formation. It was somewhat intriguing, though, that while the mannosylation process was extremely facile and unselective, the glycolipid attachment to mannosylaglycone was slow and inefficient by the actively fermenting whole cells of *K. aridum*.[6] Moreover, it was subsequently found that the mannosylating activity was almost independent from the early biosynthetic process for the heptapeptide aglycone unit and that phosphonomethylglycine (glyphosate), a potent inhibitor of aromatic amino acid biosynthesis,[8] suppressed the antibiotic production but not the mannosylating activity.

Since these findings suggest a possibility that the simple glycosylations such as mannosylation may occur in the outer surface of the cytoplasmic membrane of the producing organism, and may be non-selective in terms of the substrate structure, we have examined the possible glycosylation of aridicins, their aglycone and pseudoaglycones by various microorganisms known to produce related glycopeptide antibiotics. The fermentation broths were supplemented with the aridicin substrates (100 μg/ml) 2 days after inoculation. The broth samples were taken daily, partially purified by the Sep-Pak procedure, and analyzed by HPLC. We were not successful in detecting formation of any new antibiotics in which a novel sugar moiety was incorporated onto the aridicin structure. However, an abundant mannosylating activity and some mannosidase activity were found. Results of these experiments are summarized in Table 1. As can be seen in the table, the mannosylating activity was found in those strains that produce mannose-containing glycopeptides, although not all such strains mannosylate the aridicin-derived substrates. The overall substrate specificity for mannosylation is low as anticipated. Moreover, *Streptomyces orientalis* (NRRL 2450) and *S. candidus* (NRRL 3218)

were found to convert the mannosylaglycone of aridicin to the aglycone itself in a reasonable efficiency ($t_{\frac{1}{2}} \simeq 2$ days).

When the fermentation broth of *A. teichomyceticus* (ATCC 31121), which was supplemented with aridicins (100 μg/ml) for the possible attachment of *N*-acetylglucosamine, was kept on the shaker beyond 5~7 days, aridicins were slowly converted to a new compound with a higher hydrophilicity. The new compound has been identified as deacylated aridicin on the basis of FAB mass spec'ral analysis (*m/z* 1,633, MH^+) and isoelectric point measurement (pI 4.9 vs. pI of aridicin 3.8). Similarly, the AAD-609 antibiotics[6] were also deacylated to provide the corresponding deacyl-609 antibiotic: FAB-MS *m/z* 1,619 (MH^+); pI 7.0. This microbial deacylation turned out to be the only viable way of selectively removing the fatty acid side chain of these antibiotics. Upon over-fermentation, *A. teichomyceticus* did not appear to deacylate the teicoplanins, its own antibiotics.

Next, we examined the possibility of employing permeabilized whole cells and protoplasts of *K. aridum* in the biosynthetic studies with an objective of locating the site of the mannosylating enzyme and improving the bioconversion of mannosylaglycone to the aridicin antibiotics. One possible reason for the low efficiency observed for the conversion of mannosylaglycone to aridicin may be that whereas the mannosylation takes place in the outer surface of the cytoplasmic membrane, the processes involved with the glycolipid attachment perhaps occur deeper inside the membrane, areas not readily accessible to the exogenously added precursors. The ability to study biosynthesis of large molecules in the intact cells is frequently hindered by permeability barriers. In recent years, however, several methods have been developed to make cells more permeable to exogenous substrates; for example, treatment with organic solvents and cold-shock treatment. Toluene, phenethyl alcohol, ether and some detergents have been commonly used as the membrane permeabilizing agents.[9~11]

A number of experiments were carried out in order to evaluate the effect of toluene on the cytoplasmic membrane of *K. aridum*. The ^{14}C-labeled aridicin aglycone (5.2 μCi) was fed to the fermentation broth of *K. aridum* at 30 hours after inoculation. After fermenting for 4 days, the culture was harvested by removing mycelia by centrifugation. The mycelia were exhaustively washed ($\times 10$) with aqueous acetonitrile (50: 50) until the washing did not show significant counts of radioactivity, and then treated with toluene - H_2O (1: 5) for 2 minutes on a vortex mixer. The radioactivity counting of the various fractions showed that 72% of the radioactivity was present in the extracellular supernatant and acetonitrile - water washings, and 28% in the toluenized aqueous fraction. It is not clear whether the radioactivity in the aqueous toluene fraction represents the intracellular material or the tightly membrane-bound extracellular material. However, the observation that the bioconversion of aridicin aglycone to mannosylaglycone was completely suppressed by addition of toluene (10 μl/ml) or phenethyl alcohol (2 μl/ml) seems to suggest the mannosylation process to be cytoplasmic membrane associated.

Despite numerous efforts, the possibility of improving the bioconversion efficiency of mannosylaglycone to aridicins could not be convincingly demonstrated by cell-permeabilization with toluene, phenethyl alcohol or detergents such as cetyltrimethylammonium bromide and Triton X-100. The critical concentrations of these agents above which the *K. aridum* cells show either no production of the antibiotics or extensive leakage of intracellular material have been determined by HPLC assay; toluene (0.5%), phenethyl alcohol (0.2%), cetyltrimethylammonium bromide (5 mg/ml) and Triton X-100 (5 mg/ml). An extensive, HPLC-based search for intermediates such as aglycone and pseudo-aglycones both inside and outside of the fermenting *K. aridum* cells by sonic rupture of the cells at various time intervals was also unsuccessful. Evidently, these species, even though appearing to be

Fig. 2. Photomicrographs of *K. aridum*.
(A) Whole cells in water and (B) protoplasts in P-solution. At ×1,250 magnification on a Zeiss photomicroscope II.

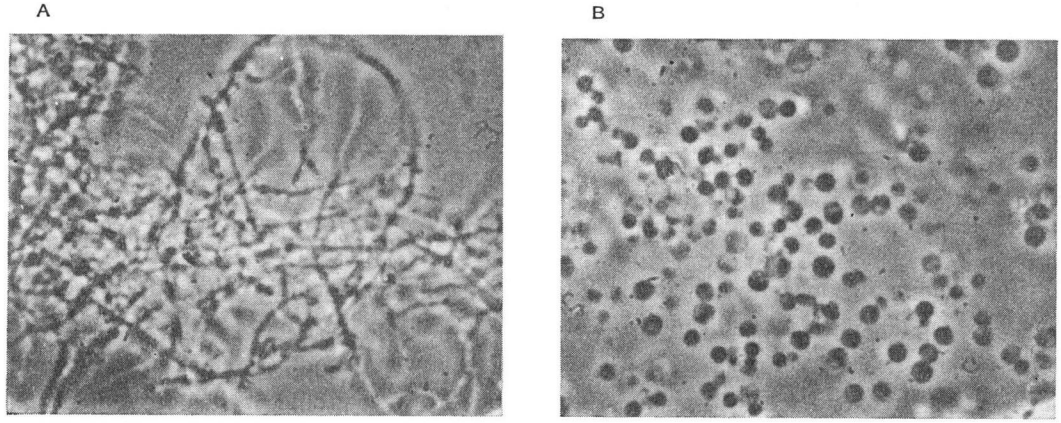
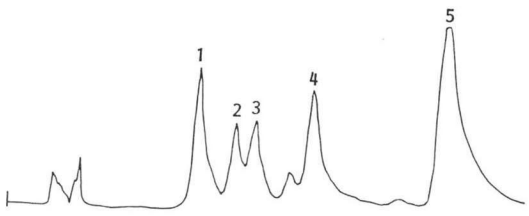


Fig. 3. HPLC trace of the protoplast products.
Beckman Ultrasphere ODS column (10×250 mm at 6 ml/minute), run isocratically with 18% CH_3CN in 0.01 M KH_2PO_4 (pH 3.2). 1. X_1, 2. X_2, 3. X_3, 4. mannosylaglycone, 5. aglycone.



true biosynthetic intermediates, do not accumulate to a detectable concentration (*ca.* 1 μg/ml). Several attempts to glycosylate aridicin aglycone in a cell-free preparation of *K. aridum* have been equally unfruitful (FARE, L.; S. K. CHUNG & Y. OH: unpublished results).

Our studies with protoplasts of *K. aridum* turned out to be much more interesting. Thus, when a protoplast preparation was incubated with aridicin aglycone in P-solution, facile glycosylations of the substrate was observed in *ca.* up to 55% yield. The viable protoplasts were prepared from *K. aridum* culture grown in TSB containing 1.5% glycine by lysozyme treatment and differential centrifugation. Photomicrographs of whole cells and protoplasts of *K. aridum* are shown in Fig. 2. The protoplasts in P-medium were found to swell 3~4 times the initial size during the usual incubation period (2 days at 30°C), but to remain stable up to 72 hours. The protoplasts were also found to readily regenerate on sucrose agar medium containing Mg^{2+}, Ca^{2+} and oatmeal.[12]

The freshly prepared protoplasts of *K. aridum* were found to retain the mannosylating ability of the whole cells, readily converting aridicin aglycone to mannosylaglycone. In addition, the protoplast preparation was capable of producing at least 3 more products, X_1, X_2 and X_3, when supplemented with aridicin aglycone. A representative HPLC trace for the bioconversion of aridicin aglycone in the protoplasts is shown in Fig. 3. A protoplast incubation with ^{14}C-labeled aglycone followed by preparative HPLC isolation and the radioactivity counting of the products clearly indicated that these products were indeed derived from the aglycone (Table 2). The chemical nature of these novel, aglycone-derived products were examined by FAB mass spectral analysis and isoelectric focusing. Based on these data, the new compounds, X_1, X_2 and X_3, are tentatively identified as aridicin aglycone derivatives in which 3, 3 and 2 units of neutral hexoses, respectively have been incorporated. These data, however, do not allow the elucidation of the sites and pattern of the novel glycosylation.

Table 2. Bioconversion of aridicin aglycone in protoplasts of *K. aridum*.

Product	Chemical yield[a] (%)	Radiochemical yield (%)	*m/z* (MH^+)[b]	pI[c]
X_1	16.7	15.3	1,782	5.3
X_2	6.0	7.6	1,782	5.3
X_3	10.7	11.9	1,620	5.2
Mannosylaglycone	21.8	22.9	1,458	5.0
Aglycone	44.8	42.3	1,296	5.0

[a] Based on the HPLC integrations.
[b] FAB-MS. Corresponding *m/z* values for MNa^+ have also been observed.
[c] Measured essentially according to a published procedure.[13]

Incubation of the mannosylaglycone with the protoplast preparation did not produce the X compounds, suggesting that mannosylation was not involved as the first step in the multiple glycosylation of the substrate. The fact that protoplasts rather than the new incubation conditions were responsible for the novel glycosylation activity could be readily shown by a control incubation of the whole cells in P-medium, which indicated an extremely slow conversion of the aglycone to mannosylaglycone ($t_{\frac{1}{2}} \simeq 7$ days) and no formation of the X compounds.

Supplementation of various sugar and glycolipid precursors into the protoplast incubation of aridicin aglycone did not bring about any discernible change to the transformation pattern described above. These precursors include glucose, glucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylgalactosamine, *N*-acetylmuramic acid, UDP-*N*-acetylglucosamine, 2-deoxy-2-[(1-oxodecyl)-amino]-α-D-glucopyranosiduronic acid[4] and the corresponding glucopyranose,[4] and *n*-decanoyl-SCoA at the 100~500 μg/ml levels. The possibility of additional glycosylation of aridicin A, AAD-609 A and other glycopeptide antibiotics by the protoplast preparation has also been investigated without success.

Other osmotic stabilizers such as sorbitol (0.8 M) and mannitol (0.8 M) instead of the sucrose component in the P-medium have been examined and found to have no dramatic effect on the glycosylation of aridicin aglycone. However, the glycosylating activity of the protoplast system was found to be effectively inhibited by various chemical agents such as DMSO (1%), decanoic acid (150 μg/ml), tunicamycin (100 μg/ml), NaN_3 (0.1 mM), sodium arsenate (0.1 mM) and inorganic phosphate (0.05 M).* The aeration rate was also found to be critical to the efficient glycosylation by the protoplasts. Incubation at 65~100 spm seemed to be optimal, whereas no glycosylation was observed at the agitation rate of 30 spm or less. It is reasonable to suggest that the inhibition by DMSO and decanoic acid is probably due to their ability to interfere with the protoplast membrane while the inhibition by NaN_3 and sodium arsenate is related to the uncoupling of the energy metabolism. The latter suggestion is also consistent with the observed aeration effect on the glycosylation. On the other hand, the glycosylating activity was found to be substantially enhanced by addition of dithiothreitol (0.1 mM). The precise reason for this observation is not obvious. The presence of Mg^{2+} and Ca^{2+} ions are known to prevent the leakage and to help the protoplast stability.[11,14,15] In the case of *K. aridum*, it was found that the presence of either one of these divalent ions could support the glycosylation activity in the protoplasts.

* Inorganic phosphate (5×10^{-2} M) added to fermentation broths was also found to completely suppress the production of the aridicin antibiotics, and to inhibit mannosylations of aridicin aglycone and demannosyl-aridicin A.

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