HYBRID BIOSYNTHESIS OF DERIVATIVES OF PROTYLONOLIDE AND M-4365 BY MACROLIDE-PRODUCING MICROORGANISMS†

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Biotransformation of a macrolide antibiotic and a related compound was studied using various macrolide-producing microorganisms grown in the presence of cerulenin, an inhibitor of *de novo* synthesis of the aglycone moiety. Protylonolide (**1**) was transformed into 5-*O*-(4′-*O*-propionylmycarosyl)protylonolide (**2**) by a leucomycin-producing strain, *Streptoverticillium kitasatoensis* KA-429. M-4365 G_2 (**3**) was bioconverted into M-4365 G_3 (**4**), 9-dihydro M-4365 G_3 (**5**), 3-*O*-acetyl M-4365 G_3 (**6**) and 3-*O*-acetyl-9-dihydro M-4365 G_3 (**7**) by a spiramycin-producing strain, *Streptomyces ambofaciens* KA-1028. Forosaminylated derivatives of M-4365 G_2 were not obtained using this microorganism. M-4365 G_2 was converted into 3-*O*-acetyl M-4365 G_2 (**8**) by *Stv. kitasatoensis* strain KA-429 and a carbomycin-producing strain, *S. thermotolerans* KA-442.

These results suggest that the substrate specificity of mycaminose- and forosamine-binding enzymes is high in *Stv. kitasatoensis* and *S. ambofaciens*, respectively, while that of the 3-hydroxyl acylating enzyme and mycarose-binding enzyme is low in these microorganisms. The bioconversion products showed lower antibacterial and antimycoplasmal activities than those of M-4365 G_2.

Many attempts have been made to obtain new antibiotic derivatives by adding the analogues of biosynthetic intermediates into the culture of an antibiotic producer or its mutant strains. The compounds thus obtained are utilized to study structure-activity relationships of the antibiotic. When an idiotroph, which requires the intermediate for the antibiotic biosynthesis, is used, this technique is called mutasynthesis or mutational biosynthesis.[1]

In a previous paper,[2] we reported the hybrid biosynthesis of 5-*O*-desosaminylprotylonolide (identical with the antibiotic M-4365 G_1) by adding protylonolide, a product of a mutant of a tylosin-producing strain,[3] to the culture of a producer of picromycin, a macrolide with desosamine as its sugar moiety. Cerulenin was added to the growing culture to inhibit *de novo* synthesis of the aglycone moiety of picromycin.

Using the technique of hybrid biosynthesis, five new bioconversion products were obtained from protylonolide or from antibiotic M-4365 G_2, a product of *Micromonospora capillata* MCRL 0940,[4] by three macrolide-producing microorganisms, *Streptoverticillium kitasatoensis*** KA-429 (a leucomycin producer), *S. ambofaciens* KA-1028 (a spiramycin producer) and *S. thermotolerans* KA-442 (a carbomycin producer) grown in the presence of cerulenin. The present paper describes the isolation, structures and biological activities of the new conversion products. The results are discussed with regard to the substrate specificity of the enzymes involved in the macrolide biosynthesis.

† Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XXIII. Part XXII of this series appeared in: S. ŌMURA, N. SADAKANE & H. MATSUBARA; Chem. Pharm. Bull. 30: 223～230, 1982

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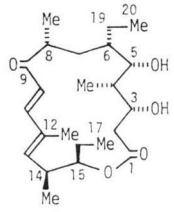
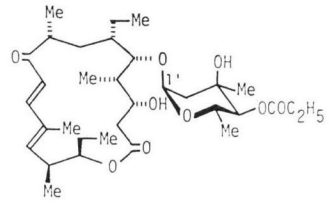
** The former name *Streptomyces kitasatoensis*[5] is revised hereafter according to the recent proposal.[6]

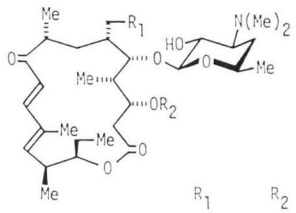
Fig. 1. Structures of protylonolide, M-4365 G_2 and their bioconversion products.



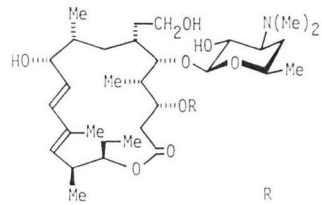
Protylonolide (1)



5-*O*-(4'-*O*-Propionylmycarosyl) protylonolide (2)



	R_1	R_2
M-4365 G_2 (3)	CHO	H
M-4365 G_3 (4)	CH_2OH	H
3-*O*-Acetyl M-4365 G_3 (6)	CH_2OH	$COCH_3$
3-*O*-Acetyl M-4365 G_2 (8)	CHO	$COCH_3$



	R
9-Dihydro M-4365 G_3 (5)	H
3-*O*-Acetyl-9-dihydro M-4365 G_3 (7)	$COCH_3$

Results and Discussion

Bioconversion of Protylonolide by *Stv. kitasatoensis*

The compound obtained from protylonolide (**1**) with a leucomycin-producing strain, *Stv. kitasatoensis* KA-429, in the presence of cerulenin was determined to be 5-*O*-(4′-*O*-propionylmycarosyl)protylonolide (**2**) on the basis of mass, ^1H and ^{13}C NMR spectral data. The ^{13}C NMR spectrum of **2** indicates the presence of an ester carbonyl at C-1 (δ 174.4) and a sugar attached to the oxygen atom at C-5 (δ 79.4) on the lactone ring. The mass fragmentation pattern showed that the sugar at C-5 was 4-*O*-propionylmycarose.

It is suggested that *Stv. kitasatoensis* KA-429 cannot attach mycaminose at C-5 nor oxidize the C-20 carbon on protylonolide. In the biosynthesis of its own antibiotic leucomycin, however, the two reactions are carried out on the aglycone moiety, platenolide,[7] followed by the attachment of mycarose to mycaminose. Accordingly, strain KA-429 biosynthesized **2** from mycarose and a foreign aglycone, protylonolide, by their direct linkage, a process which is not normally involved in leucomycin biosynthesis.

We reported the isolation of mycarosylprotylonolide[8] from a culture of a mutant strain, No. 261, of the tylosin-producing strain, *S. fradiae* KA-427. This finding and the present results indicate that the substrate specificity of the mycaminose-binding enzyme is high with respect to the aglycone, whereas that of the mycarose-binding enzyme is low in *Stv. kitasatoensis* and *S. fradiae*. The propionylation of mycarose in **2** is worth noting, since this strain produces leucomycins A_1 and A_3 as major components, both of which have an isovaleryl group at the C-4″ position of mycarose. The corresponding propionyl derivatives are minor components.

Bioconversion of M-4365 G_2 by *S. ambofaciens*

The structures of the bioconvertants of M-4365 G_2 obtained in the culture of *S. ambofaciens* in the

Table 1. ^{1}H NMR parameters of protylonolide, M-4365 G_2 and their bioconvertants.

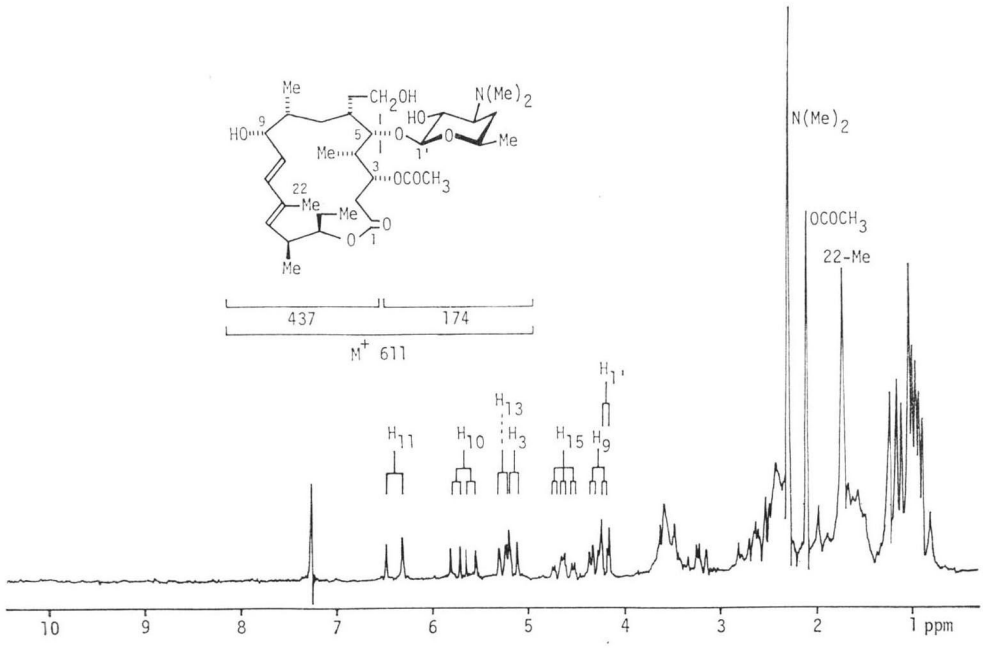
Proton	**1**	**2**	**3**	**4**
H-3	3.68, d, J=12	obscure	3.85, d, J=10	obscure
H-5	3.74, d, J=12	obscure	3.68, d, J=10	obscure
H-9	—	—	—	—
H-10	6.25, d, J=16	6.28, d, J=16	6.29, d, J=16	6.29, d, J=16
H-11	7.24, d, J=16	7.18, d, J=16	7.32, d, J=16	7.30, d, J=16
H-13	5.59, d, J=10	5.65, d, J=10	5.66, d, J=10	5.64, d, J=10
H-15	4.70, dt, J=4, 10	4.67, bt	4.72, dt, J=4, 10	4.71, dt, J=4, 10
-CHO	—	—	9.74, s	—
22-CH_3	1.79, s	1.79, s	1.78, s	1.78, s
-$OCOCH_3$	—	—	—	—
H-1′	—	4.67, bs	4.20, d, J=7	4.30, d, J=7
-$N(CH_3)_2$	—	—	2.25, s	2.40, s

Proton	**5**	**6**	**7**	**8**
H-3	3.82, d, J=10	5.20, d, J=10	5.16, d, J=10	5.15, d, J=9
H-5	3.68, d, J=10	obscure	3.5, d	3.49, d, J=10
H-9	4.27, dd, J=4, 9	—	4.29, dd, J=4, 9	—
H-10	5.68, dd, J=9, 16	6.28, d, J=16	5.67, dd, J=9, 16	6.29, d, J=16
H-11	6.16, d, J=16	7.42, d, J=16	6.39, d, J=16	7.41, d, J=16
H-13	5.18, d, J=10	5.70, d, J=10	5.24, d, J=10	5.73, d, J=10
H-15	4.69, dt, J=4, 10	4.70, dt, J=4, 10	4.64, dt, J=4, 10	4.58, dt, J=4, 10
-CHO	—	—	—	9.67, s
22-CH_3	1.73, s	1.78, s	1.74, s	1.80, s
-$OCOCH_3$	—	2.10, s	2.11, s	2.09, s
H-1′	4.30, d, J=7	4.30, d, J=7	4.20, d, J=7	4.19, d, J=7
-$N(CH_3)_2$	2.33, s	2.35, s	2.29, s	2.25, s

^{1}H NMR spectra were measured in $CDCl_3$ solutions. Chemical shift values in ppm, relative to internal TMS. ^{1}H-^{1}H coupling constants in Hz.

presence of cerulenin are shown in Fig. 1. The structures of these bioconvertants were deduced from the mass, UV and ^{1}H NMR spectral data (Table 1). In the ^{1}H NMR spectrum of **7** (Fig. 2), the signal at δ 9.74 due to the aldehyde group at C-20 which is observed in the spectrum of **3** was not seen, while a doublet at δ 5.16, a singlet at δ 2.11 and a double of doublets at δ 4.29 were observed. The signal at δ 5.16 was assigned to the methine proton at the 3-position which shifts to a lower magnetic field due to the acetylation of the C-3 hydroxyl group. The signal at δ 4.29 can be assigned to the proton at the C-9 bearing a hydroxyl group. The configuration at C-9 was determined to be *R* from the *J* value ($J_{9,10}$= 9 Hz).[9] Moreover, the EI-mass spectrum of **7** showing the molecular ion peak at *m/z* 611 and the aglycone peak at *m/z* 437 strongly indicates that the structure of **7** is 3-*O*-acetyl-9-dihydro M-4365 G_3. The structures of compounds **4**, **5** and **6** were determined by comparison of their behavior in ^{1}H NMR spectrometry and mass fragmentation. The structures were confirmed by comparison with corresponding authentic samples, prepared by acetylation and/or reduction of M-4365 G_2, using silica gel TLC and mass spectroscopy. This experiment was attempted with the hope to obtain new forosaminylated derivatives of M-4365 G_2. However, the desired compounds were not obtained. It is speculated that the forosamine-binding enzyme is highly specific in *S. ambofaciens*.

Fig. 2. ^1H NMR spectrum (90 MHz, $CDCl_3$) and mass fragmentation pattern of 3-*O*-acetyl-9-dihydro M-4365 G_3 (**7**).



Compounds **4**~**7** were found to possess a hydroxyl group at the C-20 position. The results are in agreement with the previous finding[10] that tylosin was bioconverted into 9-dihydrotylosin and 9,20-tetrahydrotylosin by *S. ambofaciens*. Spiramycin was not converted to the alcohol derivative by the spiramycin-producing strain under the same condition. Further, no compound related to the spiramycin family has been reported to possess a hydroxyl group at the C-20 position. It is obvious from the results that *S. ambofaciens* possesses a macrolide aldehyde-reducing enzyme. The enzyme acted on the aldehyde group of M-4365 G_2 and tylosin, but apparently not on that of its own antibiotic spiramycin. The biosynthetic significance of this enzyme is not known. The difference among macrolides in the susceptibility to the enzyme may be due to the strict substrate specificity of the enzyme, or to the facile permeation of foreign macrolides through the cell membrane of *S. ambofaciens* as compared with that of spiramycin.

Compounds **6** and **7** have an acetyl group at the C-3 position. This is in agreement with the results of our previous cell-free studies,[11] which indicated that the substrate specificity of the C-3 hydroxyl acylating enzyme is low in *S. ambofaciens*.

Bioconversion of M-4365 G_2 by *Stv. kitasatoensis* and *S. thermotolerans*

M-4365 G_2 was converted to its 3-*O*-acetyl derivative (**8**) by a leucomycin-producing strain, *Stv. kitasatoensis* KA-429 and a carbomycin-producing strain, *S. thermotolerans* KA-442. Of the 16-membered macrolide antibiotics, many antibiotics belonging to the leucomycin group have an acyl group attached at the C-3 position, while no members of the tylosin group of antibiotics do[12]. These latter include M-4365, tylosin, rosaramicin, angolamycin and B-58941. It is worth noting that the hydroxyl group at C-3 of M-4365 G_2 was acylated by *Stv. kitasatoensis*, *S. thermotolerans* and *S. ambofaciens*, all of which produce antibiotics of the leucomycin group. It is likely that an acylase is present in the

Table 2. Antibacterial activities of M-4365-related compounds.

Test organism	MIC (μg/ml)					
	3	**4**	**5**	**6**	**7**	**8**
Staphylococcus aureus FDA 209P	0.2	6.25	50	1.56	0.78	0.4
Bacillus subtilis PCI 219	0.78	12.5	>100	6.25	6.25	0.4
Micrococcus luteus PCI 1001	<0.2	0.4	6.25	0.78	0.4	<0.2
Mycobacterium smegmatis ATCC 607	>100	100	>100	>100	50	>100
Escherichia coli NIHJ	0.78	3.13	25	12.5	12.5	3.13
Klebsiella pneumoniae PCI 602	0.4	0.78	6.25	6.25	3.13	1.56
Proteus vulgaris IFO 3167	3.13	25	>100	12.5	6.25	6.25
Pseudomonas aeruginosa P-3	25	100	>100	100	>100	>100

MIC were determined by the agar dilution method in Nutrient agar medium (37°C, 20 hours).

producers of the leucomycin group of antibiotics, but not in the producers of the tylosin group of antibiotics. However, such an acylase is produced by various non-macrolide producing microorganisms.[12] The possibility is not ruled out at present that the tylosin-producing microorganism possesses a macrolide 3-hydroxyl acylase, which does not act on its own antibiotic, but does act on foreign macrolide antibiotics, as is the case with the aldehyde-reducing enzyme of *S. ambofaciens*.

In the experiments described in a previous section, protylonolide was converted to a derivative with a sugar at the C-5 position, but not to a derivative with an acyl group at the C-3 position. The results described above showed that such 3-acylated derivatives were obtained from 5-*O*-desosaminyl antibiotics. In view of these findings, it is likely that the acylation of the C-3 hydroxyl group requires the presence of a (amino) sugar moiety at C-5 on the 16-membered lactone ring.

Biological Activity of the Conversion Products

Table 2 summarizes the antimicrobial activity of the bioconvertants obtained. Protylonolide and its propionylmycarosyl derivative were inactive. The convertants of M-4365 G_2 were active to the same or lesser extent as compared with that of the parent compound **3**. Compound **8** showed relatively potent activity. It was compared with **3** in antimycoplasmal activity. As shown in Table 3, acetylation at C-3 of **3** resulted in a decrease of the antimycoplasmal activity. A similar decline of *in vitro* antimicrobial activity was reported on 3-*O*-acyl derivatives of leucomycins,[13] although 3-*O*-acetyltylosin and tylosin showed the same extent of activity.[14] Since the leucomycin components acylated at C-3 show higher serum levels than those of corresponding non-acylated components, further experiments are needed for the therapeutic evaluation of these convertants.

Table 3. Antimycoplasmal activities of M-4365 G_2 (**3**) and 3-*O*-acetyl M-4365 G_2 (**8**).

Test organism	MIC (μg/ml)	
	3	**8**
Mycoplasma gallisepticum KP-13	0.05	0.1
M. gallisepticum S-6	0.05	0.1
M. gallisepticum 333P*	>50	>50
M. pneumoniae	≦0.01	0.05
Acholeplasma laidlawii (A) PG-8	0.05	0.1
A. laidlawii (B) Bm-1	0.02	0.1

* Spiramycin-resistant

MIC were determined by the broth dilution method in PPLO broth containing 20% horse serum (37°C, 7 days).

Hybrid biosynthesis utilizes enzyme inhibitors in place of idiotrophs, and permits various combinations of antibiotic producers and inhibitors. Hybrid biosynthesis follows reasonable biosynthetic routes present in the microorganisms employed. As described in the present paper, the hybrid products provide information con-

cerning not only the structure-activity relationships of the antibiotics but also the properties of the enzymes involved in the biosynthetic reactions. Such information will be useful for manipulating genetic recombination techniques to elaborate new antibiotics in the near future.

Experimental

Materials

Protylonolide was obtained by fermentation using a blocked mutant, *Streptomyces fradiae* KA-427 No. 261, as described in a previous paper.[3] Cerulenin was obtained from a fermentation broth of *Cephalosporium caerulens* KF-140 by a new method (manuscript in preparation). M-4365 G_2 was obtained from Tanabe Seiyaku Co.

Analysis

The microbial transformation was monitored with a Shimadzu CS-920 chromatogram scanner. UV spectra were measured with a Shimadzu UV-210A double-beam spectrophotometer. Mass spectrometric analysis was carried out by means of a JEOL JMS-D 100 spectrometer. ^1H and ^{13}C NMR spectra were recorded with a Varian EM-390 spectrometer and a JEOL PS-100 spectrometer, respectively, with TMS as the internal standard.

Bioconversion of Protylonolide by *Stv. kitasatoensis* KA-429

A seed culture was prepared by culturing *Stv. kitasatoensis* KA-429 (66–14-3B) in a seed medium (2.0% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dried yeast, 0.5% NaCl, 0.3% $CaCO_3$, pH 7.0) for 2 days at 27°C. The seed culture (2.0% of inoculum size) was transferred into a 500-ml Sakaguchi flask containing 100 ml of a leucomycin production medium (2.0% glucose, 0.5% peptone, 0.1% meat extract, 0.5% yeast extract, 0.3% NaCl, 0.5% $CaCO_3$, pH 7.0). To the culture medium was added 40 μg/ml of cerulenin initially and at every 24-hour interval to prevent the *de novo* synthesis of the lactone moiety. After 8 hours of cultivation, 50 μg/ml of protylonolide was added and the cultivation was continued for a further 64 hours. The cultured broth was centrifuged to remove mycelia and the supernatant was extracted with an equal volume of benzene. The benzene layer was concentrated. The residue was purified by preparative silica gel TLC developed with $CHCl_3$ - MeOH - conc. NH_4OH (20: 1: 0.05). Fifteen mg of a white powder of 5-*O*-(4′-*O*-propionylmycarosyl)protylonolide (**2**) was obtained from 25 liters of the culture to which 1.25 g of protylonolide was added. The physicochemical properties of **2**; mass: *m/z* 594 (M^+), 538 (M^+ −propionyl+H^+), 393 (aglycone), 377 (aglycone−O), 57 (propionyl), ^{13}C NMR: δ 174.4 (C-1), δ 204.1 (C-9), δ 96.8 (C-1′), δ 172.4 (C-8′).

Bioconversion of M-4365 G_2 by *S. ambofaciens* KA-1028

S. ambofaciens strain KA-1028 (ISP 5053) was cultured in a spiramycin production medium (1% glucose, 1% dried yeast, 0.1% $NaNO_3$, 0.5% NaCl, 1% $CaCO_3$, pH 7.5) in the presence of cerulenin. After 24 hours, 50 μg/ml of M-4365 G_2 was added into the culture and the cultivation was continued for a further 48 hours. The cultured broth was centrifuged to remove mycelia and the bioconvertants in the supernatant fluid were extracted with benzene. The benzene extracts were purified by silica gel column chromatography ($CHCl_3$ - MeOH - conc. NH_4OH, 10: 1: 0.05) followed by preparative TLC on silica gel with the same solvent system to afford M-4365 G_3 (**4**), 9-dihydro M-4365 G_3 (**5**), 3-*O*-acetyl M-4365 G_3 (**6**) and 3-*O*-acetyl-9-dihydro M-4365 G_3 (**7**). From 12 liters of the culture to which 600 mg of M-4365 G_2 was added, compounds **4** (7.1 mg), **5** (13.5 mg), **6** (6.6 mg) and **7** (13.4 mg) were obtained. Physicochemical properties of these compounds are as follows. M-4365 G_3 (**4**); UV: λ_{max} nm (ε) 282.5 (16200), Mass: *m/z* 567 (M^+), 393 (aglycone), 174 (desosaminyl). 9-Dihydro M-4365 G_3 (**5**); UV: λ_{max} nm (ε) 234 (21500), Mass: *m/z* 569 (M^+), 395 (aglycone), 174 (desosaminyl). 3-*O*-Acetyl M-4365 G_3 (**6**); UV: λ_{max} nm (ε) 282.5 (11800), Mass: *m/z* 609 (M^+), 435 (aglycone), 174 (desosaminyl). 3-*O*-Acetyl-9-dihydro M-4365 G_3 (**7**); UV: λ_{max} nm (ε) 234 (19900), Mass: *m/z* 611 (M^+), 437 (aglycone), 174 (desosaminyl).

Bioconversion of M-4365 G_2 by *S. thermotolerans*

S. thermotolerans strain KA-442 (KCC S-0159) was cultured in a carbomycin production medium

(1% glucose, 0.5% peptone, 0.5% soybean meal, 0.3% $CaCO_3$, pH 7.0) in the presence of cerulenin. After 48 hours, 80 μg/ml of M-4365 G_2 was added and the cultivation was continued for a further 24 hours. The cultured broth (2.5 liters) was extracted with benzene and the benzene extracts were subjected to silica gel column chromatography ($CHCl_3$ - MeOH - conc. NH_4OH, 15: 1: 0.05) to give a white powder (91 mg) of 3-*O*-acetyl M-4365 G_2 (**8**). The physicochemical properties of **8**; Mass: *m/z* 607 (M^+), 433 (aglycone), 174 (desosaminyl), ^{13}C NMR: δ 170.0 (C-1), δ 203.7 (C-9), δ 204.0 (C-20), δ 171.0 (–*O*C*O*CH$_3$), δ 104.8 (C-1′).

Bioconversion of M-4365 G_2 by *Stv. kitasatoensis* KA-429

Strain KA-429 was cultured under the same condition as those used in the bioconversion of protylonolide by the organism, and 145 mg of M-4365 G_2 was added to the culture, from which 12.5 mg of **8** was obtained.

Preparation of M-4365 G_2-related Compounds

M-4365 G_3 and 9-dihydro M-4365 G_3 were obtained by reduction of M-4365 G_2 with $NaBH_4$ in MeOH - 0.2 M phosphate buffer (pH 7.5) (1: 1) for 13 hours at room temperature. 3-*O*-Acetyl M-4365 G_2 was prepared by acetylation of **3** in acetic anhydride and pyridine (1: 1) at room temperature overnight followed by solvolysis in MeOH. 3-*O*-Acetyl M-4365 G_3 and 3-*O*-acetyl-9-dihydro M-4365 G_3 were obtained by reduction of **8** with $NaBH_4$ in MeOH - 0.2 M phosphate buffer (pH 7.5) (1: 1) for 19 hours at room temperature followed by extraction with $CHCl_3$ and purification by preparative TLC ($CHCl_3$ - MeOH - conc. NH_4OH, 10: 1: 0.05).

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