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Microbial hydroxylation of rustmicin (galbonolide A) and galbonolide B, two antifungal products produced by *Micromonospora* sp.

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Abstract

In order to synthesize derivatives of galbonolide A and B with improved chemical stability and antifungal activity profiles, a panel of microorganisms consisting of various species of actinomycetes and fungi were screened. As a result, an organism, *Streptomyces halstedii*, was identified, which catalyzed the formation of two polar compounds, one from each of the galbonolides. The synthesis and the relative stability of these compounds were optimized by using washed cells, which had been prepared from the transforming organism, and reaction conditions, which included the usage of MES buffer with pH adjusted to 5.5 and incubation at 27°C. Under conditions thus established, two compounds were isolated and characterized by a combination of UV, mass, and NMR spectroscopic analysis. The data indicate the synthesis of 21-hydroxy derivatives of galbonolides A and B with reduced but still significant anti-fungal activity when compared to the parent galbonolides.

Keywords: Rustmicin (galbonolide A); Galbonolide B; *Micromonospora* sp.

1. Introduction

In our laboratories, enzymatic and microbial catalytic potentials are exploited for the structural modification of known chemicals, particularly, natural products with useful therapeutic properties. Galbonolide A (rustmicin) and B [Fig. 1] are two related polyketide natural products that were initially isolated by German and Japanese scientists [1,2]. These compounds were recently isolated from a *Micromonospora* sp. at Merck [3] where total synthesis and crystal structure for galbonolide B [4,5] and absolute stereochemistries for galbonolide A and B [4,6] have been determined. In light of their impressive biological activity, these two compounds were introduced into a microbial biotransformation program in order to evaluate the synthesis of novel derivatives with improved chemical and biological properties. We now report the production, isolation and characterization of the 21-hydroxylated deriva-

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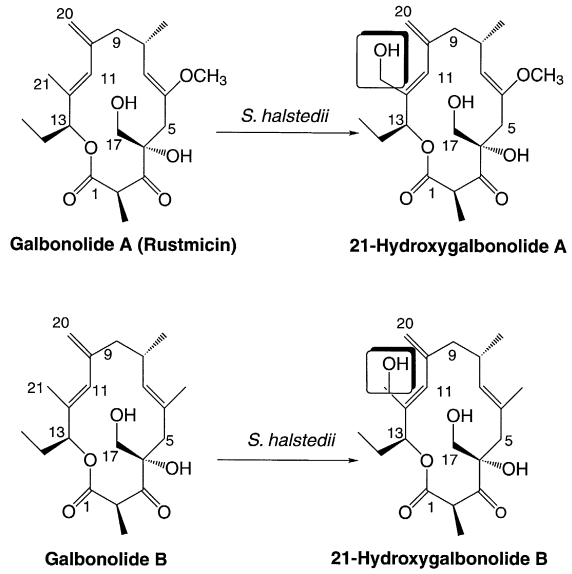

Fig. 1. Chemical structures of galbonolides A and B and their respective biotransformation products.

tive of both galbonolides, which were synthesized by a *Streptomyces* sp., identified as *Streptomyces halstedii*. Subsequently, these compounds were also isolated from a large-scale fermentation broth of the galbonolides-producing organism as minor components, with both showing potent anti-fungal activities. The biosynthetic implication of the 21-hydroxylated derivatives on the biochemical characterization of the galbonolides biosynthetic pathway will also be discussed.

2. Experimental

2.1. Preparation of biotransformation cultures

Cultures under investigation were grown under our standard conditions in seed and biotransformation media. The seed medium consisted of: 0.1% dextrose, 1% dextrin, 0.3% beef extract, 0.5% ardamine pH, 0.5% NZ amine type E, 0.005% $MgSO_4 \cdot 7H_2O$, 0.037% K_2HPO_4, and 0.05% $CaCO_3$ with pH adjusted to 7.1 before autoclaving. Biotransformation medium contained: 2% glucose, 0.5% soya meal, 0.5% yeast extract, 0.5% NaCl, 0.98% MES with pH adjusted to 7.0 before autoclaving.

2.2. Biotransformation screening

Biotransformation of galbonolide A and B were carried out using either fermentation cultures or their washed cells as described below.

2.2.1. Fermentation cultures

In this method, frozen seed cultures or isolated colonies that were stored on solid agar plates, were used for the inoculation of the seed medium. Usually, 2 ml of the seed or loopful of cultures were inoculated into 250-ml plain Erlenmeyer flask containing 50 ml of the seed medium. These cultures were incubated at 27°C on a shaker with 220 rpm gyratory agitation. After 40 h of incubation, 2 ml of the seed cultures were transferred into 50 ml of the biotransformation medium along with a solution of either galbonolide A or B (2.5 mg/0.5 ml DMSO) in 250-ml baffled Erlenmeyer flask. Incubation of these cultures was continued for 40 h under identical conditions as described above for the seed cultures. At different time intervals, a sample was withdrawn from each flask and mixed with an equal volume of methanol. After vortexing and centrifugation, an aliquot of the resulting supernatant was then analyzed for the formation of a new product by HPLC using an analytical reverse-phase C_8 column (Zorbax) at room temperature, under an isocratic solvent system consisting of methanol:0.025 M ammonium acetate, pH 4.5 (75:25) with a flow rate of 30 ml/h.

2.2.2. Washed cells

For washed cells preparation, fermentation cultures, as described above, were harvested by centrifugation on a Beckman table top centrifuge at 3750 rpm for 15 min. The pellet was then washed three times by suspension in 0.1 M MES [2-(*N*-morpholine)ethane sulfonic acid] buffer, pH 5.5, followed by centrifugation. The washed pellet was then used for biotransformation, or stored at −80°C for future use, according to the following procedures. A total of 10 g (wet weight) of washed cells, prepared from each screening culture, was suspended in 0.1 M

MES buffer, pH 5.5, and the final volume was brought to 30 ml. One milligram of compound under investigation was dissolved in 0.1 ml of methanol and was added to each flask containing the cell suspension before incubation under conditions as described above. At different time intervals, a sample was withdrawn from each flask and analyzed by HPLC as before.

2.3. Isolation, characterization and antifungal activity of the 21-hydroxy-derivatives of galbonolide A and B

Isolation, characterization and antifungal properties of the material eluted with newly formed HPLC peaks from the biotransformation extracts were carried out according to the previously reported procedures [3]. Briefly, methylene chloride extracts were worked up and the dried residues of the extracts were purified on a HPLC column. The structures of the isolated fractions were determined by UV (Beckman model DU70 spectrophotometer), MS (Jeol SX-102A, electron impact, EI, 90eV and TSQ700 for electronspray ionization) and proton NMR (500MHz Varian Unity) spectroscopic analysis.

3. Results and discussion

3.1. Stability studies and time course of the formation of 21-hydroxy analogs of galbonolide A and B

Achenbach et al [7] previously studied the chemical stability of both galbonolides and suggested that they were most stable at pH 5.5 in buffered aqueous solution. Based on this information, we initially carried out our experiments with the pH of the biotranformation medium adjusted to 5.5. Despite poor growth of many of the screening cultures, in addition to the loss of compounds under investigation, one culture, *S. halstedii*, was identified, which gave rise to the formation of a new HPLC peak after 16 h of incubation [Fig. 2]. As shown in this figure, this peak eluted from the column with a retention time of 9.64 min, as compared with the retention time of the galbonolide A, which eluted at 22.10 min. Furthermore, as shown in the same figure, the peak representing the elution of the galbonolide A nearly disappeared after 40 h of incubation; similar results were obtained for galbonolide B. In order to circumvent the problems of the chemical stability and the poor growth, which were observed with many of the

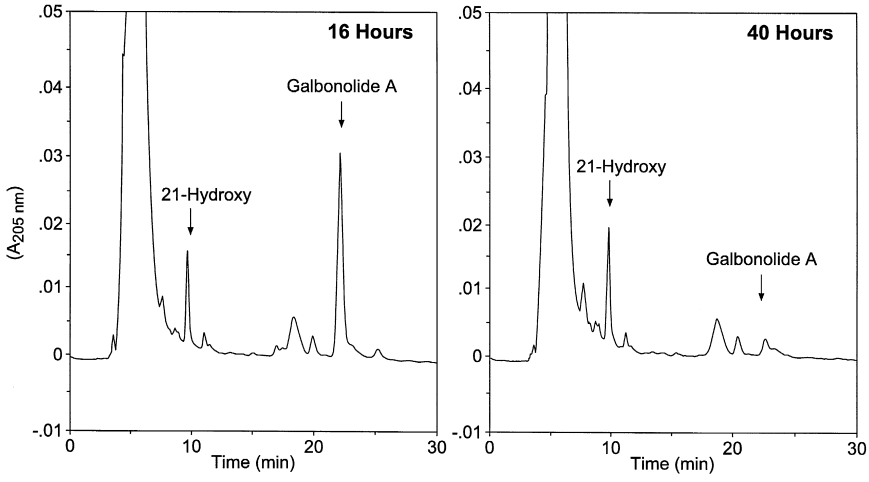

Fig. 2. HPLC elution profiles of galbonolide A and its biotransformation product from the C_8 reverse-phase column during the time course of the bioconversion in the actively growing culture of *Micromonospora* sp. In this HPLC system, galbonolide A and its 21-hydroxy derivative elute at 9.64 and 22.10 min, respectively.

cultures, washed cells that had been prepared under normal pH condition from screening microorganisms were utilized for transformation. As shown in Fig. 3, the UV-absorption of the HPLC peaks from the extracts of the washed cell suspension system for both 21-hydroxy derivatives were more intense than the similar peaks arising from the extracts of the whole broths. In light of this observation, subsequent screenings were therefore carried out with washed cells and 21-hydroxy derivatives of galbonolide A and B were synthesized and isolated for both structure elucidation and antifungal activity evaluation.

3.2. Characterization and antifungal activities of 21-hydroxy derivatives of galbonolide A and B

The molecular ion of the compound that was isolated from the HPLC peak with 9.64 retention time [Fig. 2] was observed by ESI as the sodium adduct at m/z 419 (M + Na)] indicating a MW of 396. High-resolution EI-MS data was obtained on M-H_2O at m/z 378. The empirical formula obtained for this ion was $C_{21}H_{30}O_6$ based on found mass of 378.2028 (calculated 378.2042) for $C_{21}H_{32}O_7$–H_2O. This corresponds to a molecular formula of $C_{21}H_{32}O_7$ for isolated material indicating a possible hydroxylation of galbonolide A (MW = 380, $C_{21}H_{32}O_6$), which occurs with displacement of a proton. Proton NMR of this material in C_6D_6 gave signals at δ 0.837 (t, 7.0, H-15), 0.908 (d, 7.0, H-19), 1.382 (d, 7.0, H-16), 1.67 (m, H-14), 1.840 (dd, 11.2, C-17 OH), 1.939 (brt, 5.2, C-21 OH), 2.112 (dd, 2.9, 13.2, H-9a), 2.274 (d, 14.9, H-5a), 2.354 (dd, 7.7, 13.2, H-9b), 2.485 (d, 14.9, H-5b), 2.93 (m, H-8), 3.172 (s, H-18), 3.342 (dd, 3.1, 11.9, H-17a), 3.572 (dt < 1, 12, H-17b), 3.713 (q, 7, H-2), 3.822 (s, C-4 OH), 4.126 (dd, 4.9, 12.7, H-21a), 4.488 (dd, 4.4, 12.6, H21b), 4.671 (d, 9.4, H-7), 4.829 (brs, H-20a), 4.908 (brs, H-20b), 5.226 (t, 7.3, H-13), 6.117 (brs, H11). The proton signals as indicated here are similar to signals obtained for galbonolide A except for H21a and H21b at 4.126 and 4.488 ppm, which have replaced the signal at 1.69 ppm. This observation in conjunction with the mass data indicates hydroxylation of C-21, which, as expected, has given rise to the down field shift of the C-21 methyl protons in galbonolide A.

Similarly, the structure of the material eluting at 11.45 min [Fig. 3] was determined to be 21-hydroxy derivative of galbonolide B. This compound gave

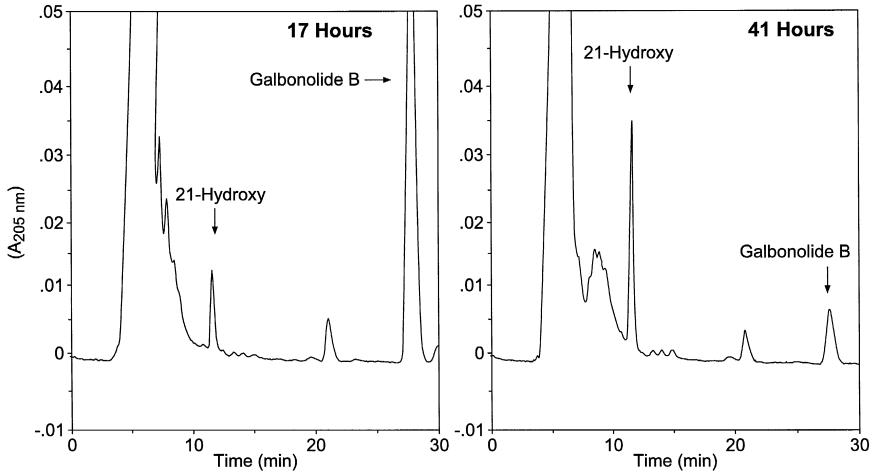

Fig. 3. HPLC elution profiles of galbonolide B and its derivative from the C_8 reverse-phase column during the time course (17 and 18 h) of the bioconversion in the washed cells suspension. In this HPLC system, galbonolide B and its 21-hydroxy derivative elute at 11.45 and 27.64 min, respectively.

molecular ion of 380.2187 in HREI-MS (calc. 380.2199), suggesting a molecular formula of $C_{21}H_{32}O_6$ with proton NMR signals as follows: δ 0.844 (d, 6.5, H-19), 0.858 (t, 7.5, H-15), 1.408 (d, 7, H-16), 1.639 (brs, H-18), 1.680 (m, H-14), 1.852 (d, 14, H-5), 2.110 (dd, 7.5, 13, H-9), 2.198 (brd, 12, H-9), 2.43 (m, H-8), 2.674 (d, 14, H5), 3.276 (d, 11.5, H-17), 3.468 (brt, 10.5, H-17), 3.776 (dq, 6.5, H-2), 4.102 (d, 12.5, H-21), 4.558 (d, 12.5, H-21), 4.805 (brs, H-20), 5.166 (brd, 9.5, H-7), 5.230 (brt, 7.0, H-13), 6.117 (brs, H-11). As for the first compound, the addition of 16 atomic mass unit along with the down field shifts of the two C-21 protons established the 21-hydroxylation of the galbonolide B, which has MW of 364 with a molecular formula of $C_{21}H_{32}O_6$.

The two 21-hydroxy compounds that were prepared by biotransformation of the galbonolide A and B showed significant antifungal activity against a number of pathogenic fungi including *Candida* sp. and *Cryptococcus neoformans*. The results were identical to the antifungal activity of the similar compounds, which were subsequently isolated from the galbonolides producing *Micromonospora* sp. [3].

3.3. Implication of the isolation of 21-hydorxy derivatives on the biochemical and genetic analysis of galbonolides biosynthesis

As a result of extensive research efforts on the genetics and enzymology of macrolide antibiotics biosynthesis, vast amounts of information have become available in recent years. For example, it is now clear that the macrocycle moiety of erythromycin, a 14-membered macrolide antibiotic, is formed by the condensation of six methylmalonates with one propionate as a starter unit [8]. This suggests that galbonolides, which, like erythromycin, are 14-membered macrolides, are also formed by the condensation of five methylmalonates, one acetate (C_5–C_6) and one propionate as a starter unit [Fig. 4]. Despite this fundamental similarity in the biosynthesis of the macrocycle cores in erythromycin and galbonolides, the presence of a diene system in galbonolides (C_{10}–C_{11}) creates a structural difference between these compounds. Formation of this diene system cannot be explained by simple reduction and dehydration processes as suggested for other macrolides in that the positions of the unsaturations in galbonolides do not fit the standard macrolide biosynthetic schemes. In view of the fact that two 21-hydroxylated derivatives of the galbonolides were isolated in our biotransformation studies and these derivatives were also isolated from the galbonolides producing *Micromonospora* cultures, we conclude that the formation of the diene system in the galbonolides are probably triggered by the initial hydroxylation of the macrocycle moieties after completion of the macrocycle biosynthesis. Furthermore, we propose that these hydroxylations are carried out by cytochrome *P*450 systems, which have been known to carry out such oxidative reactions both in biosynthesis [9] and in the biotransformation of xenobiotics by microorganisms [10]. Needless to say, the mechanism of the formation of the diene system after proposed initial hydroxylation remains to be determined.

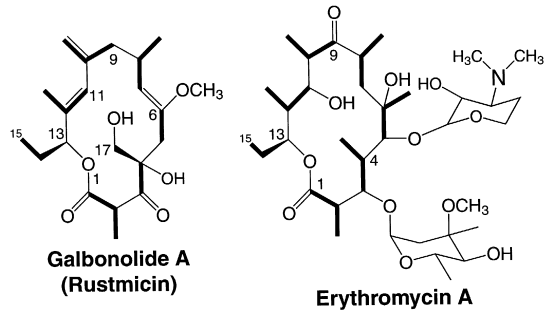

Fig. 4. Chemical structures of galbonolide A and erythromycin A in which methylmalonates and acetate condensing units are shown by solid heavy lines. Propionate starting units (C_{13}–C_{15}) are also shown by solid heavy lines in both structures.

References

[1] H. Achenbach, A. Muhlenfeld, U. Fauth, H. Zaehner, Tetrahedron Lett. 26 (1985) 6167.
[2] Y. Abe, H. Nakayama, A. Shimazu, K. Furihata, K. Ikeda, H. Seto, N. Otake, J. Antibiot. 38 (1985) 1810.
[3] G. Harris, A. Shafiee, M.A. Cabello, J.E. Curroto, O. Genilloud, K.E. Goklen, M.B. Kurtz, M. Rosenbach, P.M. Salmon, R.A. Thornton, D.L. Zink, S.M. Mandala, J. Antibiot. 51 (1998) 837.
[4] B. Tse, J. Am. Chem. Soc. 118 (1996) 7094.

[5] Unpublished results from Dr. R. Ball of the Merck Crystallography Group.
[6] B. Tse, C.M. Blazey, B. Tu, J. Balkovec, J. Org. Chem. 62 (1997) 3236.
[7] H. Achenbach, A. Muhlenfeld, U. Fauth, H. Zaehner, Ann. N. Y. Acad. Sci. 544 (1988) 128.
[8] L. Katz, S. Donadio, Annu. Rev. Microbiol. 47 (1993) 875.
[9] A. Shafiee, R. Hutchinson, Biochemistry 26 (1987) 6204.
[10] A. Shafiee, T. Chen, P. Cameron, Appl. Environ. Microbiol. 61 (1995) 3544.