J|A|C|S COMMUNICATIONS

Published on Web 02/16/2010

Biosynthesis of Spinosyn in *Saccharopolyspora spinosa*: Synthesis of Permethylated Rhamnose and Characterization of the Functions of SpnH, SpnI, and SpnK

Hak Joong Kim, Jess A. White-Phillip, Yasushi Ogasawara, Nara Shin, Eta A. Isiorho, and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, Department of Chemistry and Biochemistry, and Institute of Cellular & Molecular Biology, University of Texas at Austin, Austin, Texas 78712

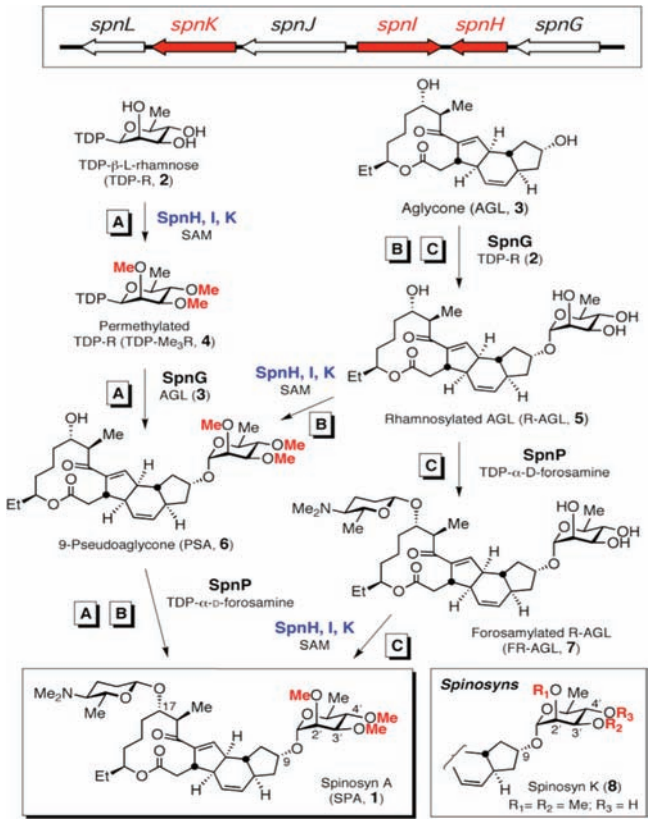
Received December 3, 2009; E-mail: h.w.liu@mail.utexas.edu

Spinosyn A (SPA, **1**) is a polyketide-derived macrolide produced by *Saccharopolyspora spinosa*, that is an active ingredient in several commercial insecticides.[1] The structures of SPA and its many analogues have a characteristic perhydro-*as*-indacene core, which is glycosylated by a rhamnose (see **2**) at C-9 and a forosamine at C-17. Both the aglycone (AGL, **3**) and the sugar appendages contribute to the observed activity of the spinosyns, among which SPA is most potent.[2] Alteration of the tetracyclic nucleus or removal of either deoxy sugars significantly diminishes the pesticidal activity. Even subtle structural variations, such as the methylation pattern of the rhamnose moiety in spinosyns, change the LD_{50} by as much as >200-fold against *Heliothis virescens*.[2a] In view of these results and the global market value of spinosyns as a general purpose insecticide, a detailed investigation of the biosynthesis of spinosyns is warranted. Since the rhamnose moiety is methylated to a different extent in various spinosyns,[3] the study of how the corresponding methyltransferases are coordinated to achieve the desired level of methylation of rhamnose has been a focus of this research. We report herein the function and substrate specificity of the three methyltransferases involved in the methylation reactions, the preferred reaction sequence of their catalyzed reactions, and the likely regulation of permethylation of rhamnose in **1**.

The spinosyn biosynthetic gene cluster had been cloned from *S. spinosa*.[4] Sequence analysis and gene disruption experiments revealed that the *spnH*, *spnI*, and *spnK* genes,[4] all of which show high sequence identity to those encoding *S*-adenosyl-L-methionine (SAM) dependent methyltransferases (MTs), are involved in the *O*-methylation of rhamnose in **1**.[4,5] As illustrated in Scheme 1, methylations may take place before (**2** → **4** → **6**, route A) or after (**3** → **5** → **6**, route B) the attachment of rhamnose to the aglycone (AGL, **3**). It is also possible that methylations are the final tailoring steps after both sugars have been coupled to the aglycone (**3** → **5** → **7** → **1**, route C). These methylation reactions are also not necessarily consecutive and may occur between the glycosylation steps catalyzed by SpnG and SpnP. To biochemically verify the functions of the MTs involved and to distinguish the possible methylation pathways, we studied the catalytic properties of the *spnH*, *spnI*, and *spnK* gene products and validated their roles in the permethylation of **1**.

The genes were amplified from *S. spinosa* genomic DNA and individually cloned into pET28b(+) expression vectors.[6] Each was subsequently heterologously expressed in *Escherichia coli* BL21 star (DE3) or Rosetta II (DE3) host cells and was purified as an *N*-terminal His_6-tagged protein by affinity chromatography using Ni-NTA resin.[6] Interestingly, the purified SpnH, SpnI, or SpnK (20 μM each), upon incubation with 1 mM of **2** and 5 mM of SAM in 50 mM Tris·HCl buffer (pH 7.5) at 30 °C, failed to methylate TDP-L-rhamnose (TDP-R, **2**), which was prepared from TDP-D-glucose as previously described via the actions of RfbB (a 4,6-dehydratase), RfbC (a 3,5-epimerase), and RfbD (a 4-reductase) from *Salmonella enterica* LT2.[7] This result clearly ruled out route A as a possible biosynthetic pathway (Scheme 1).

Scheme 1



To check whether route B is operative, the presumed substrate, R-AGL (**5**), was chemoenzymatically prepared using SpnG, which catalyzes the transfer of the rhamnose moiety from **2** to the aglycone (AGL, **3**).[8] The competence of R-AGL (**5**) as substrate for SpnH, SpnI, and SpnK was evaluated under the aforementioned assay conditions (*vide supra*).[6] In contrast to the assays with TDP-R (**2**) alone, HPLC analysis showed that all three MTs, in the presence of SAM, could convert **5** to new products (Figure 1). These new products (**I**, **II**, **III**) were isolated from preparative-scale incubation mixtures and characterized by 2-D NMR spectroscopy and high-resolution mass spectrometry.[6] As depicted in Figure 1, the regiospecificity of the methylation reaction can be readily determined by comparing the chemical shifts of the rhamnose signals in **5** versus those in the products, and the heteronuclear long-range

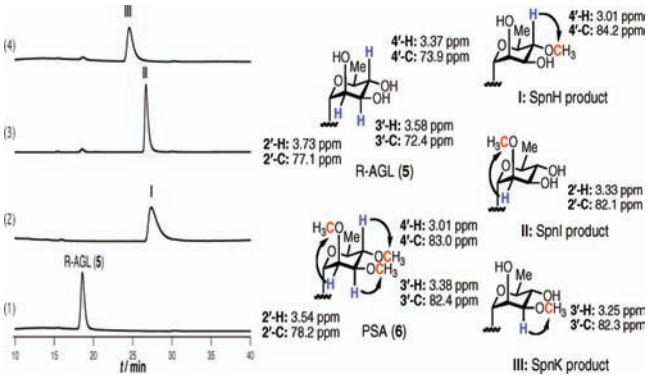


Figure 1. HPLC analysis showing formation of compounds **I**, **II**, and **III** upon incubation of (1) R-AGL (**5**) with (2) SpnH, (3) SpnI, and (4) SpnK. The pertinent chemical shifts of **I**, **II**, and **III** are shown with arrows denoting the heteronuclear long-range correlation between the methyl carbon (in red) and the nearest *O*-methine proton (in blue). See Supporting Information for details.[6]

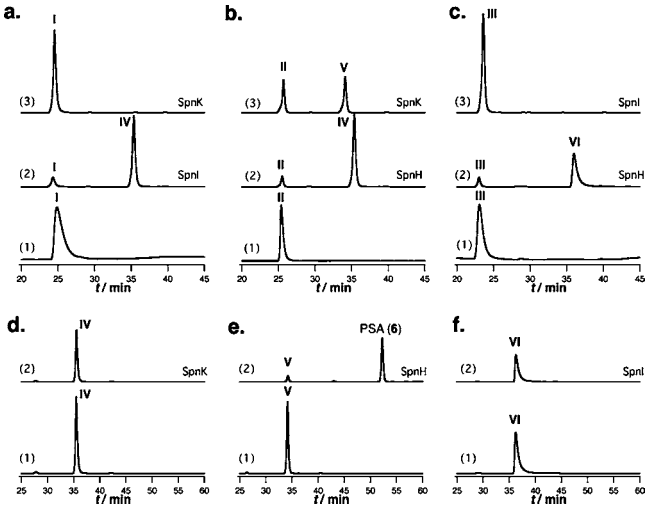
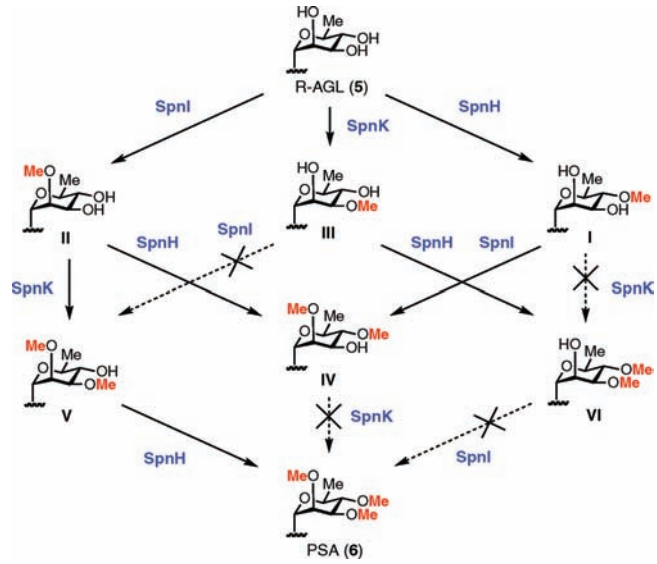


Figure 2. HPLC analysis of methyl transfer reactions using monomethylated (**I**, **II** and **III**) and dimethylated (**IV**, **V**, and **VI**) substrates. (a) Incubation of **I** (1) **I** standard, with (2) SpnI, and (3) SpnK; (b) incubation of **II** (1) **II** standard, with (2) SpnH, and (3) SpnK; (c) incubation of **III** (1) **III** standard, with (2) SpnH, and (3) SpnI; (d) incubation of **IV** (1) **IV** standard, with (2) SpnK; (e) incubation of **V** (1) **V** standard, with (2) SpnH; (f) incubation of **VI** (1) **VI** standard, with (2) SpnI.

correlations between the newly introduced methyl carbon and the nearest *O*-methine proton. These data firmly establish that **5** is a common substrate for SpnH, SpnI, and SpnK, which are the respective rhamnose 4′-, 2′-, and 3′-*O*-methyltransferase.[4,5]

Having determined that SpnH, SpnI, and SpnK recognize and methylate R-AGL (**5**), we proceeded to investigate whether these enzymes can also accept the monomethylated compounds (**I**, **II**, **III**) as substrates. As shown in Figure 2a−c, SpnH is able to modify **II** and **III** to produce the corresponding 2′,4′- and 3′,4′-di-*O*-methylated products (**IV** and **VI**) under the above assay conditions. In contrast, SpnI can methylate **I** to give **IV**, but it is unable to accept **III** as a substrate. Similarly, SpnK methylates **II** to produce the 2′,3′-di-*O*-methylated product (**V**) but fails to modify **I**. These observations show that formation of three doubly methylated products (**IV**, **V**, and **VI**) is possible and also reveal that SpnI and SpnK do not tolerate the presence of a methoxy moiety at O-3′ and O-4′ positions of the rhamnose unit, respectively. To explore whether compounds **IV**, **V**, and **VI** can be converted to the permethylated product PSA (**6**) by the appropriate MT, further experiments were carried out using these di-*O*-methylated

Scheme 2



intermediates in the incubation reactions. If the inability of SpnI and SpnK to process rhamnose carrying a methoxy group at C-3′ and/or C-4′ is an intrinsic property of these catalysts, the synthesis of PSA (**6**) can only be achieved by SpnH through intermediate **V**. As predicted (see Figure 2d−f), SpnI and SpnK were unable to process **VI** and **IV**, whereas SpnH successfully methylated the O-4′ position of **V** resulting in the formation of PSA (**6**).

The six possible biosynthetic permethylation sequences of rhamnose in **5** are summarized in Scheme 2. Because di-*O*-methylated intermediates **IV** and **VI** are not processed by SpnK and SpnI, respectively, and the mono-*O*-methylated intermediate **III** is not a substrate for SpnI, permethylation of rhamnose must begin with SpnI, followed by SpnK, and end with SpnH (**5** → **II** → **V** → **6**). Since the great majority (>95%) of spinosyns (spinosyn A and D) produced by *S. spinosa* bear permethylated rhamnose at C-9, reactions resulting in the accumulation of products with mono- and di-*O*-methylated rhamnose[3] (such as **I**, **II**, **III**, **IV**, **VI**) are likely aborted paths. In view of the occurrence of various routes leading to dead-end intermediates (such as **IV** and **VI**) *in vitro*, the action of the three rhamnose MTs are likely rigorously orchestrated during spinosyn production *in vivo* to prevent the buildup of dead-end intermediates.

The location of the encoding genes, *spnH*, *spnI* and *spnK*, of these MTs on different operons (see Scheme 1) suggests that differential expression of these MT genes may be a mechanism to regulate rhamnose methylations during spinosyn *in vivo* production and minimize the formation of dead-end intermediates. This hypothesis could be tested *in vitro* by varying the concentration of each MT in the assays.[6] As shown in Figure 3(1), when equal concentrations (20 μM) of three MTs are used in the incubation mixture, PSA (**6**) is the major product (75%). However, a significant amount of **IV** (25%) is also produced. The absence of the other dead-end intermediate, **VI**, in the reaction mixture is interesting. Since SpnK cannot process **I**, the only route leading to **VI** (**5** → **III** → **VI**) requires SpnK and SpnH working in sequence. The fact that also **III** was not accumulated suggests that this path might be prevented by the lower catalytic efficiency of SpnK toward R-AGL (**5**) than with either or both SpnH and SpnI. Formation of **IV** can instead be accomplished via two routes involving either **I** or **II** as the intermediate. In both cases, SpnH plays a key role. In fact, the competition between SpnH and SpnK for **II** could determine the flux of the reaction to either the accumulation of **IV** or the production of **V** then PSA (**6**). On the basis of the results shown in Figure 3(1) where **IV** is a significant byproduct, it was

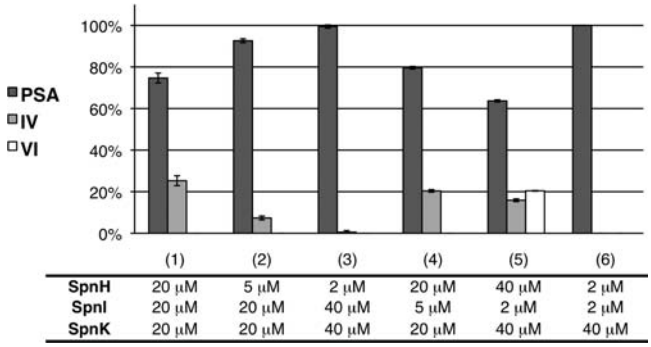


Figure 3. Product distribution change upon the variation of enzyme concentration. Experiments were performed in duplicate (error bars represent standard deviations). In all cases, the substrate, R-AGL (**5**), was completely consumed and only negligible amounts (<0.5%) of monomethylated products (**I**, **II**, **III**) were detected.

envisioned that reduction of the SpnH concentration in the incubation mixture might facilitate the formation of more PSA (**6**) than **IV** by decreasing the flux of **5** → **I** → **IV** and that of **II** → **IV**. Indeed, PSA (**6**) became almost the exclusive product when the concentration of SpnH was lowered to 2 μM and those of SpnI and SpnK were raised to 40 μM (Figure 3(2) and (3)).

Alternatively, the product composition can be altered so that the formation of dimethylated products is favored (**5** → **I** → **IV** or **5** → **III** → **VI**) by suppressing the SpnI-catalyzed conversion of **5** → **II**. Interestingly, little effect was discernible when the SpnI concentration was reduced 4-fold as compared to those of SpnH and SpnK (Figure 3(4)). It appears that the preference of SpnI toward R-AGL (**5**) is much higher than those of the other two MTs. To gain support for this idea, the SpnI concentration was reduced to 20-fold lower than that of SpnH and SpnK. As anticipated, suppression of PSA synthesis (64%) is now evident and the production of both **IV** (16%) and **VI** (20%) is also observed (Figure 3(5)). From these results, one can conclude that the majority of **IV** produced in the presence of sufficient SpnI (Figure 3(1) and (4)) is derived via the route involving **II** (**5** → **II** → **IV**). Therefore, it would also be possible to suppress the formation of **IV** by increasing the concentration of SpnK to enhance the flux from **II** to **V**. In accord with this prediction, when we increased the SpnK concentration and kept SpnH and SpnI concentrations low, the exclusive formation of PSA (**6**) was observed (Figure 3(6)).

Previously, the permethylation of rhamnose in the elloramycin pathway had also been studied. The three MTs (ElmMI, ElmMII, and ElmMIII) identified were shown to be regiospecific at O-2′, O-3′, and O-4′ of rhamnose, respectively.[9e] The order of the methylation events (ElmMI → ElmMII → ElmMIII) was also proposed. However, these results, obtained by *in vitro* cell-free-extract assays and *in vivo* deletion/complementation assays, remained to be biochemically validated. The experiments described herein unambiguously establish that SpnH, SpnI, and SpnK are the corresponding 4′-, 2′-, and 3′-*O*-methyltransferase responsible for the permethylation of rhamnose during spinosyn biosynthesis.[4,5]

Investigation of the sequence of the methylation events in the spinosyn case revealed that only one route is productive (**5** → **II** → **V** → **6**) for the permethylation of the rhamnose moiety. Moreover, the completion of rhamnose permethylation is likely achieved by the proper control of the expression levels of the methyltransferase genes involved. Specifically, it was demonstrated that the production of PSA (**6**) could be enhanced by reducing the cellular concentration of SpnH and/or by increasing the SpnK concentration. Our results also showed that the methylation reactions do not take place prior to the SpnG reaction (route A, Scheme 1) but instead occur after rhamnose has been coupled to the aglycone (route B). Recently, the heterologously expressed SpnH was shown to be capable of converting 4′-*O*-desmethyl-SPA (spinosyn K, **8**) to SPA (**1**), indicating that SpnH is the 4′-*O*-methyltransferase and route C is also feasible.[10]

The methylation of deoxysugars found in various macrolides is a common modification and known to enhance the metabolic stability and often the biological activity of the parent compounds. Several MTs responsible for such processes have been studied.[9] The permethylation reported herein represents one of the most complex *post*-glycosylation modifications of a deoxysugar in secondary metabolites. The methylation sequence is established by reconstituting the pathway with purified enzymes, and such information is otherwise inaccessible by genetic or bioinformatic experiments. Thus, this work not only provides valuable insight into the rhamnose permethylation events during spinosyn biosynthesis but also highlights the significance of the biochemical approach in deciphering natural product biosynthetic pathways. Our results set the stage for future exploitation of the spinosyn biosynthetic pathway to produce targeted spinosyn derivatives.

Acknowledgment. We thank Dr. Yung-nan Liu for technical assistance on protein purification. This work was supported by a grant from the National Institutes of Health (GM35906).

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kirst, H. A.; Michel, K. H.; Martin, J. W.; Creemer, L. C.; Chio, E. H.; Yao, R. C.; Nakatsukasa, W. M.; Boeck, L. D.; Occolowitz, J. L.; Paschal, J. W.; Deeter, J. B.; Jones, N. D.; Thompson, G. D. *Tetrahedron Lett.* **1991**, *32*, 4839–4842.

(2) (a) Sparks, T. C.; Crouse, G. D.; Durst, G. *Pest. Manag. Sci.* **2001**, *57*, 896–905. (b) Salgado, V. L.; Sparks, T. C. In *Comprehensive Molecular Insect Science*; Iatrou, K., Gill, S. S., Gilbert, L. I., Eds.; Pergamon: Oxford, 2005; Vol. 6, pp 137−173.

(3) Monomethylated: spinosyn P, W (R_1 = Me, R_2 = R_3 = H), spinosyn U, V (R_2 = Me, R_1 = R_3 = H), spinosyn T (R_3 = Me, R_1 = R_2 = H); dimethylated: spinosyn K, O, Y (R_1 = R_2 = Me, R_3 = H), J, L, M, N (R_1 = R_3 = Me, R_2 = H), spinosyn H, Q, R, S (R_1 = H, R_2 = R_3 = Me).

(4) Waldron, C.; Matsushima, P.; Rosteck, P. R., Jr.; Broughton, M. C.; Turner, J.; Madduri, K.; Crawford, K. P.; Merlo, D. J.; Baltz, R. H. *Chem. Biol.* **2001**, *8*, 487–499.

(5) SpnH, SpnI, and SpnK had previously been assigned as the respective rhamnose 4′-, 2′-, and 3′-*O*-methyltransferase based on the gene disruption results.[4] Later, in a feeding experiment using 3-*O*-rhamnosyl erythronolide and *Saccharopolyspora erythraea* mutant strains bearing various assorments of *spnH*, *spnI*, and *spnK* genes, Leadlay and coworkers demonstrated that SpnI and SpnK are the corresponding 2′- and 3′-*O*-methyltransferase. (Gaisser, S.; Lill, R.; Wirtz, G.; Grolle, F.; Staunton, J.; Leadlay, P. F. *Mol. Microbiol.* **2001**, *41*, 1223–1231). The catalytic capabilities of these methyltransferases, however, have not been tested using the natural substrates. The substrate specificity of each enzyme and the overall methylation sequence also remain unexplored.

(6) See Supporting Information for spectral data and experimental details.

(7) Borisova, S. A.; Zhang, C.; Takahashi, H.; Zhang, H.; Wong, A. W.; Thorson, J. S.; Liu, H.-w. *Angew. Chem., Int. Ed.* **2006**, *45*, 2748–53.

(8) Chen, Y. H.; Chen, Y. H.; Lin, Y. C.; Tsai, K. C.; Chiu, H. T. *J. Biol. Chem.* **2009**, *284*, 7352–7363.

(9) (a) Thibodeaux, C. J.; Melançon, C. E.; Liu, H.-w. *Angew. Chem., Int. Ed.* **2008**, *47*, 9814–9859. (b) Thibodeaux, C. J.; Melançon, C. E.; Liu, H.-w. *Nature* **2007**, *446*, 1008–1016. (c) Tylosin (mycinose, TylE/F): Kreuzman, A. J.; Turner, J. R.; Yeh, W. K. *J. Biol. Chem.* **1988**, *263*, 15626–33. (d) Mycinamicin (mycinose, MycE/F): Li, S.; Anzai, Y.; Kinoshita, K.; Kato, F.; Sherman, D. H. *ChemBioChem* **2009**, *10*, 1297–1301. (e) Elloramycin (rhamnose, ElmMI/II/III): Patallo, E. P.; Blanco, G.; Fischer, C.; Braña, A. F.; Rohr, J.; Méndez, C.; Salsa, J. A. *J. Biol. Chem.* **2001**, *276*, 18765–18774. (f) Erythromycin (cladinose, EryG): Paulus, T. J.; Tuan, J. S.; Luebke, V. E.; Maine, G. T.; DeWitt, J. P.; Katz, L. *J. Bacteriol.* **1990**, *172*, 2541–6. (g) Oleandomycin (oleandrose, OleY): Rodríguez, L.; Rodríguez, D.; Olano, C.; Braña, A. F.; Méndez, C.; Salas, J. A. *J. Bacteriol.* **2001**, *183*, 5358–63. (h) Tylosin (mycaminose, TylM1)/Methymycin (desosamine, DesVI): Chen, H.; Yamase, H.; Murakami, K.; Chang, C.-W. T.; Zhao, L.; Zhao, Z.; Liu, H.-w. *Biochemistry* **2002**, *41*, 9165–9183. (i) Spinosyn (forosamine, SpnS): Hong, L.; Zhao, Z.; Melançon, C. E., III.; Zhang, H.; Liu, H.-w. *J. Am. Chem. Soc.* **2008**, *130*, 4954–4967.

(10) Huang, K. X.; Zahn, J.; Han, L. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 1669–76.

JA910223X