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Investigation of the Origin of C_2 Units in Biosynthesis of Streptolydigin

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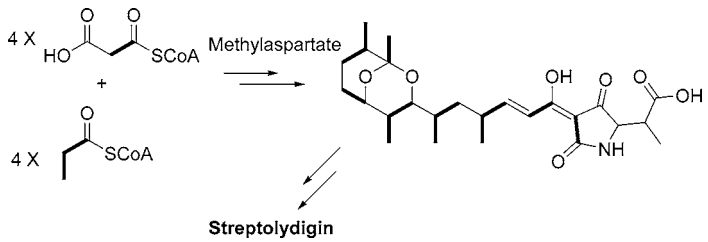
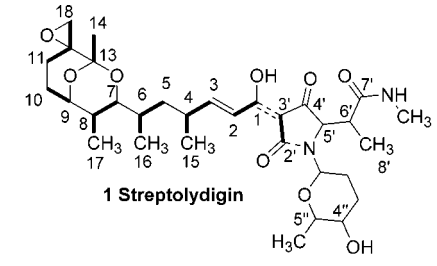
ABSTRACT



Isotope labeling studies show that malonate, not acetate, furnishes all four C_2 units in the acyltetramic acid streptolydigin. The results are compared with those for pramanicin, and implications for the biosynthetic pathway are discussed.

The acyltetramic acids (ATAs) (3-acyl-2,4-pyrrolidinediones, e.g., streptolydigin **1**, Figure 1) are a group of natural products which possess a broad spectrum of biological activity.[1] These structures, which have attracted substantial synthetic interest,[2] function as ionophores, and several are isolated as metal chelates.[3] Examples of natural products that have been proposed to be formed by modification of ATAs are also known, e.g. tenellin[4] and pramanicin **2**[5] (Figure 1).

Biosynthetically, there is substantial evidence from a range of studies that the acyl groups in ATAs derive from a polyketide, while the ring derives from a C_2 unit and an amino acid.[6] However, the order in which these three entities are joined remains ambiguous. Thus, the three bonds which are formed between these precursors (C-1−C-3′, C-2′−N-1′, and C-3′−C-4′ in **1**) could be formed in any order. For example, the side-chain acyl group could be extended to attach the ring C_2 unit prior to joining to the amino acid (Scheme 1, pathway A) or the amino acid could be



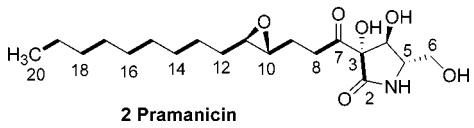


Figure 1. Structures of streptolydigin (**1**) and pramanicin (**2**).

(1) Rosen, T. *Drugs Future* **1989**, *14*, 153.
(2) For recent examples, see: (a) Detsi, A.; Micha-Screttas M.; Igglessi-Markopoulou, O. *J. Chem. Soc., Perkin Trans. 1* **1998**, 2443. (b) Detsi, A.; Markopoulos, J.; Igglessi-Markopoulou, O. *Chem. Commun.* **1996**, 1323. (c) Athanasellis, G.; Igglessi-Markopoulou, O.; Markopoulos, J. *Synlett.* **2002**, 1736. (d) Mitsos, A.; Zografos, A. L.; Igglessi-Markopoulou, O. *J. Org. Chem.* **2000**, *65*, 1201.
(3) Bhat, S. V.; Kohl, H.; Ganguli, B. N.; de Souza, N. J. *Eur. J. Med. Chem. Chim. Ther.* **1977**, *12*, 53.
(4) Wright, J. L. C.; Vining, L. C.; McInnes, A. G.; Smith, D. G.; Walter, J. A. *Can. J. Biochem.* **1977**, *55*, 678.
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(6) Royles, B. J. L. *Chem. Rev.* **1995**, *95*, 1981.

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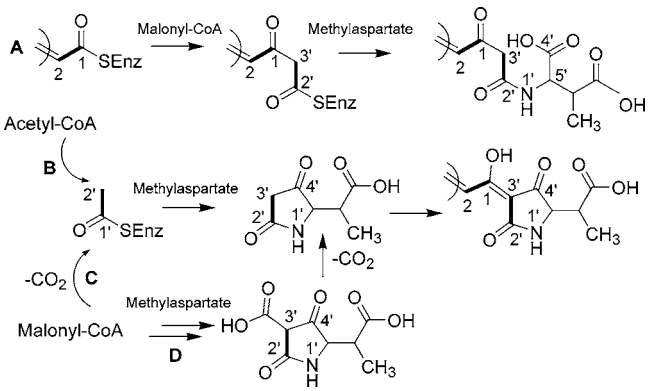
Scheme 1. Possible Pathways to the Tetramic Acid Ring en Route to Streptolydigin



N-acetylated, the ensuing acyl derivative cyclized to afford a tetramic acid intermediate, and the polyketide side chain then joined as a final step (pathways B and C).[7] In pathway D, if the decarboxylation occurs at a later step, the malonyl group is loaded onto the enzyme and then couples with methylaspartate directly; the ensuing cyclization and decarboxylation processes would generate the ring system. Path B differs in that the C_2 unit in the tetramic acid ring derives from acetate, whereas paths A, C, and D imply a malonate origin. Each of these potential routes has substantial chemical precedents.[8]

In examining the biosynthesis of pramanicin **2**, we showed that assembly starts from an acetyl unit: C-20 retains three deuterons from $D_3C^{13}CO_2Na$, while diethyl [2-^{13}C]malonate[9] labels C-20 at a lower rate than other sites, e.g., C-18.[5c] In the latter experiment, each of the even-numbered carbons of the acyl chain, C-18 to C-8, was efficiently labeled by diethyl malonate, as expected for normal cycles of chain extension on a polyketide synthase (PKS), where decarboxylative condensation of (alkyl) malonates onto the growing chain occurs. However, C-3 was labeled to the same extent as C-20, suggesting that the ring acyl unit was derived from acetate, not malonate. Since the ring acyl unit did not appear to derive from the expected precursor for PKS-based chain extension onto C-7, we suggested that pathway B seemed more likely.[5c]

Intrigued by the potential of diethyl malonate labeling to shed light on the nature of acyltetramic acid biosynthesis, we wished to test whether this phenomenon was general and chose streptolydigin **1** for this purpose. Previous biosynthetic work by Rinehart shows that streptolydigin is an acyltetramic acid containing a C_2 starter, several C_2 and C_3 extender units, and a C_2-derived ring unit.[10] However, poor incorporation of acetate coupled with the use of radio-labeled rather than ^{13}C-labeled malonate precluded detailed analysis of the origin of the ring acyl unit. Here, we describe the results of our labeling experiments.

Streptomyces lydicus was cultured and streptolydigin isolated by minor modifications of published precedures.[10a] NMR spectra were essentially as reported,[11] except that the carbonyl group carbon resonances C-1, C-2′, and C-7′, were reassigned, on the basis of modern NMR methods, especially HSQC, HMBC, and [1,2-$^{13}C_2$]acetate labeling (see below), which together gave a full and unambiguous assignment of all proton and carbon-13 resonances.

When sodium [1-^{13}C]acetate was incorporated into streptolydigin in a pulsed labeling manner we observed good incorporation by NMR, in contrast to results for single-dose incorporation.[10a] Label was found at each of the sites corresponding to the polyketide C_2 units, C-13, C-9, C-1, and C-2′, at essentially equal enrichments (3.8−4.3%). In addition, label was found at C-3, C-5, C-7, and C-11 (0.8−1.1%), sites which were also labeled by [1-^{13}C]propionate in agreement with Rinehart's study.[10a] C-4′ (2.4%) and C-7′ (7.6%) were labeled as well. These indirect incorporations are consistent with passage of the labeled acetate through the tricarboxylic acid (TCA) cycle, ultimately labeling propionate via succinate, as well as β-methylaspartate via 2-oxoglutarate. β-Methylaspartate then acts as the amino acid precursor of the tetramic acid moiety, as proposed by Rinehart.[10a] Further experiments with [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate were fully consistent with this picture, and the corresponding indirect incorporations via the TCA cycle were again observed.

Next, diethyl [2-^{13}C]malonate was administered to *S. lydicus*. Malonate was incorporated into streptolydigin very efficiently (ca. 8.8%), labeling C-3′, C-2, C-10, and C-14 with no discernible difference in enrichment levels between these four sites. However, labeling of the expected sites for indirect incorporations via acetate and the TCA cycle did not occur within limits of detection (0 ± 0.25%). Upon repeating this experiment with simultaneous addition of labeled diethyl malonate and unlabeled sodium acetate, a technique commonly used to facilitate observation of the malonate "starter" effect,[12] the overall enrichments were lower (ca. 2.6%), but again no significant differences in incorporation levels or incorporations via the TCA cycle were observed. When [1-^{13}C]acetate and [2-^{13}C]diethyl malonate were co-fed into the streptolydigin producing culture, C-3′, C-2, C-10, C-14 (ca. 3.0% each) and C-2′, C-1, C-9, C-13 (ca. 1.5% each) were labeled by malonate and acetate, respectively. Furthermore, C-3, C-5, C-7, C-11, C-4′, and C-7′ were labeled by [1-^{13}C]acetyl-CoA via the TCA cycle. No other significant incorporation (C-4, C-6, C-8, C-12, C-15, C-16, C-17, C-18, C-5′, C-6′, and C-8′) was observed at sites which would be labeled by [2-^{13}C]malonyl-CoA if it were in rapid equilibrium with acetyl-CoA.[13]

(7) For detailed discussion of several of the possible sequences and prior experimental data suggesting one or another route, see ref 6.

(8) For examples, see: (a) Neukom, C.; Richardson, D. P.; Myerson, J. H.; Bartlett, P. A. *J. Am. Chem. Soc.* **1986**, *108*, 5559. (b) Kohl, H.; Bhalt, S. V.; Patell, J. R.; Ghandi, N. M.; Nazareth, J.; Divekar, P. V.; de Souza, N. J.; Fehlhaber, H.-W. *Tetrahedron Lett.* **1974**, 983.

(9) For an example where malonate is shown to incorporate selectively into extender units, see: Chandler, M. I.; Simpson, T. J. *J. Chem. Soc., Chem. Commun.* **1987**, 17.

(10) (a) Pearce, C. J.; Ulrich, S. E.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1980**, *102*, 2510. (b) Pearce, C. J.; Rinehart, K. L., Jr. *J. Antibiot.* **1983**, *36*, 1536.

(11) Lee, V. J.; Rinehart, K. L., Jr. *J. Antibiot.* **1980**, *33*, 408.

(12) (a) Birch, A. J.; Cassera, A.; Rickards, R. W. *J. Chem. Soc., Chem. Commun.* **1961**, 654. (b) Bardshiri, E.; Simpson, T. J. *Tetrahedron* **1983**, *39*, 3539.

Thus, malonate labels only the C_2 units of streptolydigin, whereas acetate labels the C_2 units as well as C_3 units and the amino acid moiety. Hence, acetyl-CoA gives malonyl-CoA but not vice versa, and starter unit C-13−C-14 and terminal unit C-2′−C-3′ are derived directly from malonyl-CoA and not via acetyl-CoA.

To further confirm the above results, a feeding experiment with [1-^{13}C,$^{2}H_3$]acetate was carried out. As anticipated, C-2′, C-1, C-9, and C-13 were enriched efficiently (ca. 4.7% each) through the standard polyketide pathway. Meanwhile, lower but significant enrichments (ca. 1.2% each) at C3, C-5, C-7, C-11, C-4′, and C-7′ were also observed, again via the TCA cycle. However, no isotopically shifted signals were oberved for C-1, C-9, and C-13, suggesting an extensive deuterium exchange process during the polyketide chain extension. However, it is well-known that some carbon atoms such as carbonyl carbons can be poor "reporter" atoms for β-^{2}H shifts, leading to a zero shift.[14] The presence of the ^{2}H label was then checked by direct ^{2}H NMR analysis (Figure 2).

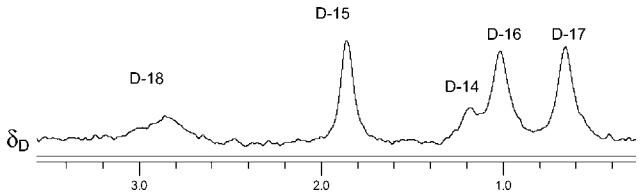


Figure 2. Deuterium NMR spectrum showing deuterium incorporation from [1-^{13}C,$^{2}H_3$]acetate.

Deuterium incorporations were observed at positions 15, 16, 17, and 18, indicating at least partial retention of deuterium in acetate through the TCA cycle. A notable observation was the lower level (1:3 integral ratio compared with positions 15, 16 and 17) of deuterium enrichment at position 14 which corresponds to the starter unit.

To determine the numbers of deuterium atoms retained at C-14, [2-^{13}C,$^{2}H_3$]acetate was incorporated. The triple-resonance probe technique,[15] which allows simultaneous decoupling of both ^{1}H and ^{2}H while the ^{13}C resonance is being observed, was used to analyze the resultant streptolydigin. Deuterium was observed by shifted singlets in the ^{13}C NMR spectrum which appeared only when ^{2}H was decoupled: ^{13}CDH (C-18) at 50.7 ppm ($\Delta\delta$ = 0.33 ppm); $^{13}CDH_2$ and $^{13}CD_2H$ at 22.3 ppm (C-14) ($\Delta\delta$ = 0.25 ppm/D) (Figure 3); $^{13}CDH_2$ and $^{13}CD_2H$ at 17.3 ppm (C-16) ($\Delta\delta$ = 0.28 ppm/D); and $^{13}CDH_2$ and $^{13}CD_2H$ at 12.3 ppm (C-15) ($\Delta\delta$ = 0.26 ppm/D). The shifted signals at C-17 were not visible due to overlap with other resonances. Most notably, no signal for retention of all three deuterons from the labeled acetate precursor was observed.

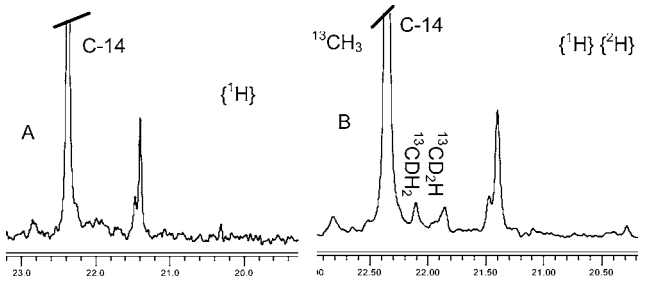


Figure 3. Partial ^{13}C NMR spectra showing C-14 of **1** derived from [2-^{13}C,$^{2}H_3$]acetate: A, proton decoupled; B, proton and deuterium decoupled.

These results, coupled with the extensive washout of deuterium, seen in the incorporation of ^{13}C without any deuterium attached, are consistent with the requirement that acetate must pass through malonate to furnish the starter unit.

In modular (type I) polyketide synthases (PKSs), multifunctional proteins responsible for the biosynthesis of macrolides and other complex polyketides,[16] a distinct feature is the presence of a loading module,[17] which is composed of an acyltranferase (AT) and an acyl carrier protein (ACP) domain. In some PKSs, e.g., 6-deoxyerythronolide B synthase, monocarboxylic acyl-CoA species such as acetyl-CoA or propionyl-CoA are used as starter units. However, other modular PKS proteins contain a loading module that possesses an additional modified ketosynthase (KS) called KS^Q,[18] which is similar to the chain length factor (CLF) of type II PKSs. Both KS^Q and CLF are similar to KS, except that the active site cysteine has been mutated into a glutamine (Q) residue. Such domains typically recruit dicarboxylic acyl-CoAs such as malonyl-CoA as starter units.

The present results indicate that the loading module for streptolydigin PKS contains a specific domain, which catalyzes decarboxylation of malonyl-CoA that is recruited from the intracellular pool by the AT of the loading module. A KS^Q-like domain is clearly a candidate for this reaction.

Malonate was also found to be the origin of the C-2′−C-3′ unit. Clearly, pathway B (Scheme 1) is excluded. Therefore, there are three possibilities for the formation of the tetramic acid ring. First, the C-1−C-3′ bond is formed initially by a standard polyketide chain extension process, then the product couples with methylaspartic acid to form the N-1′−C-2′ and the C-3′−C-4′ bonds (Scheme 1, path A). On the other hand, the N-1′−C-2′and/or C-3′−C-4′ bonds

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(14) Simpson, T. J. *Top. Curr. Chem.* **1998**, 195.
(15) Vederas, J. C. *Nat. Prod. Rep*, **1987**, *4*, 277.

(16) For recent reviews, see: (a) Rawlings, B. J. *Nat. Prod. Rep.* **2001**, *18*, 190. (b) Rawlings, B. J. *Nat. Prod. Rep.* **2001**, *18*, 231. (c) Staunton J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380.
(17) (a) Pedera, A.; Summers, R. G.; Stassi, D. L.; Ruan, X.; Katz, L.; *Microbiology* **1998**, *144*, 543. (b) Long, P. F.; Wilkinson, C. J.; Bisang, C. P.; Cortës, J.; Dunster, N.; Oliynyk, M.; McCormic, E.; McArthur, H.; Mendez, C.; Salas, J. A.; Staunton, J.; Leadlay, P. F. *Mol. Microbiol.* **2002**, *43*, 1215. (c) Brautaset, T.; Borgos, S. E. F.; Sletta, H.; Ellingsen, T. E.; Zotchev, S. B. *J. Biol. Chem.* **2003**, *17*, 1493.
(18) (a) Bisang, C.; Long, P. F.; Cortës, J.; Westcott, J.; Crosby, J.; Matharu, A. L.; Cox, R. J.; Simpson, T. J.; Staunton, J.; Leadlay, P. F. *Nature* **1999**, *401*, 502. (b) Wilkinson, J.; Frost, E. J.; Staunton, J.; Leadlay, P. F. *Chem. Biol.* **2001**, *8*, 1197.

could be formed prior to acylation by the side-chain intermediate (Scheme 1, path C and D), but only if C-2′−C-3′ derives from malonyl-CoA. It has been shown that the epothilone PKS has a KS(Y) loading module that generates an acetyl-enzyme from malonyl-CoA; the acetyl group is in turn transferred to the active site of module 1.[19] Therefore, an analogous process would generate *N*-acetylmethylaspartate prior to cyclization to afford the tetramic ring, with the C-acylation of the ring by the polyketide side chain occurring last to give the carbon skeleton of streptolydigin. Alternatively, malonate reacts with methylaspartate first and decarboxylation occurs in a later step (path D). Interestingly, these results are in direct contrast to the proposed pathway (path B) to pramanicin based on the evidence that the C_2 unit in the ring has an acetate origin. Whether this contrast is due to the pramanicin pathway not involving an ATA intermediate, or perhaps to differences in organisms that produce these compounds, bacterium and fungus, respectively, is unclear and remains the subject of further study.

In summary, feeding experiments with malonate and acetate have shown that the starter unit in streptolydigin biosynthesis originates from malonate, suggesting a KS^Q-like loading module in the PKS. Furthermore, the C_2 unit in the tetramic acid ring derives from malonate, and a pathway in which methylaspartate is *N*-acetylated by acetyl-CoA is thus excluded. Hence, either the side chain polyketide is extended by malonate prior to addition of methylaspartate, or methylaspartate is acylated by malonyl-CoA specifically. The results show that in this organism, acetate feeds into the malonate pool, but not vice versa. Thus, mixed incorporations of [1-^{13}C]acetate and [2-^{13}C]malonate provide a useful technique to elucidate the presence or absence of rapid equilibration between these species in studies of polyketide biosynthesis.

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Supporting Information Available: Experimental procedures, ^{2}H NMR spectrum of streptolydigin derived from [1-$^{13}CD_3$]acetate incorporation and ^{1}H NMR spectrum for chemical shift comparison, and table of incorporation rates. This material is available free of charge via the Internet at http://pubs.acs.org.

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