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# The Biosynthesis of Coloradocin

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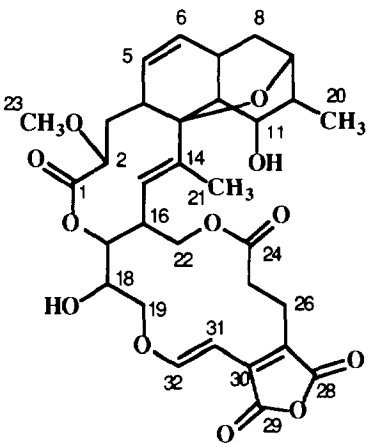
**Abstract:** The biosynthesis of coloradocin involves assembly of a polyketide chain similar to those of nargenicin and nodusmicin and an apparent non-polyketide eleven carbon unit which incorporates acetate and succinate (but not propionate) by some undetermined pathway. Precursor incorporation was studied using single and double labeled C-13 precursors and label scrambling was suppressed by the simultaneous addition of other unlabeled substrates. Copyright © 1996 Elsevier Science Ltd

## *INTRODUCTION*

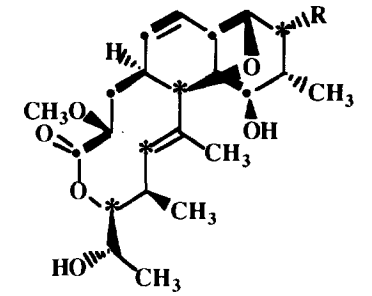
The ability of microorganisms to construct complex molecules from simple precursors is unexcelled. After a generation of study many important details of the molecular events involved have been revealed and in many cases the precursors and even the pathways can be predicted accurately in advance of experimentation. Many of the more intriguing secrets which remain can be approached by experimental study of molecules whose structures defy such facile biogenetic prediction. Coloradocin appealed to us as a subject of study based upon these considerations and we report herein our preliminary findings.

Coloradocin[1], an antigonorrheoal and antianaerobic antibiotic produced by fermentation of *Actinoplanes coloradoensis* sp. nov, was subsequently found to be identical to luminamicin[2] from an apparent *Nocardioides sp.*. Its chemical structure was found to consist of an unprecedented pentacyclic macrodilactone skeleton, one ring of which is a rare maleic anhydride moiety. Much of the stereochemistry of coloradocin remains to be elucidated.

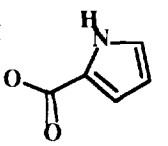
The closest previously known analogs containing the upper tricyclic lactone ring are nargenicin[3] and nodusmicin[4]. The biosynthesis of nargenicin and nodusmicin have been studied[5] and found to follow the typical polyketide pattern being derived from five acetate, four propionate and one methionine-derived methyl unit. It was reasonably proposed that the octahydronaphthalene ring was derived from an internal Diels-Alder carbocyclization reaction subsequent to laying down of the skeleton itself. An enzyme catalysed Diels-Alder reaction has been conclusively demonstrated for the formation of the octalin ring system in the fungal metabolite, solanapyrone.[6] Cane and coworkers[7] have demonstrated the incorporation of intact polyketide chain elongation intermediates (fed as *N*-acetylcysteamine thiol esters) up to and including a pentaketide, into nargenicin. The oxidation state of these intermediates is consonant with the biosynthetic model involving a Diels-Alder cyclization. Additional post-polyketide assembly events in the biosynthesis of nargenicin resulted in the formation of a bridging ether ring, introduction of the methoxyl moiety and esterification with pyrrole-2-carboxylic acid in an undetermined order. Both ether oxygens as well as the alcohol at C-18 have their origin in molecular oxygen.[5b] Understanding of polyketide biosynthesis has advanced dramatically in recent years mainly from genetic and biochemical studies.[8] Although this work has not extended to nargenicin or nodusmicin, for other macrolides, notably erythromycin and avermectin, the genes encoding the polyketide synthases have been characterized. They occur as a series of *orf*s comprising two or three modules each of which encodes the enzymes required for a condensation and the appropriate modification of the ß-ketoester formed.[8] A modified polyketide synthase from the first *orf* of 6-deoxyerythronolide B synthase (the polyketide synthase of erythromycin biosynthesis) has been isolated and developed into a functional cell-free system.[9]



COLORADOCIN



NODUSMICIN R = OH

NARGENICIN A1 R = 

= $CH_3COO^-$ derived

= $CH_3CH_2COO^-$ derived

Our working hypothesis for coloradocin biosynthesis at the outset was that the upper tricyclic ring of coloradocin was derived in the same general manner as those of nargenicin and nodusmicin with some differences in that the ester moiety is not present, the ether ring was closed at C-9 instead of C-8, and hydroxylation occurred at C-19 and C-22. The newly installed C-19 and C-22 oxygens enable installation of the lower bicyclic ring, a prominent feature absent from nargenicin/nodusmicin. The origin of the carbons of the lower bicyclic ring was unclear. A polyketide origin could be proposed however the branchings are atypical and would require invoking some unusual reactions, particularly where the C-27 to C-30 linkage was installed. Further, anhydride rings are comparatively rare in natural products and have seldom been the subject of biosynthetic studies. Cantharidin has been shown to have a mevalonate origin through farnesol.[10] The antibiotics, tautomycin and tautomycetin have maleic anhydride moieties derived from propionate and a-ketoglutarate.[11] The monomeric C-9 maleic anhydride postulated[12] as the precursor to the nonadrides is assembled from hexanoic acid and oxaloacetate as demonstrated by $^{14}$-C incorporation studies.[13] None of these structures possessed the appropriate carbon chain and/or oxidation states to suggest that it was a good model for the lower ring of coloradocin. It was these unusual features of coloradocin that provided the primary motivation for us to undertake the study reported here.

## *RESULTS AND DISCUSSION*

In order to provide unequivocally the needed spectral dispersion, NMR measurements were made in deuterated methylene chloride solution rather than in deuterated dimethylsulfoxide or deuterochloroform, the solvents utilized in the structural studies. The original peak assignments were confirmed by fresh $^1$H(300 MHz) and $^{13}$C-(125 MHz), $^1$H-$^{13}$C and $^1$H-$^1$H correlation measurements (500 MHz), DEPT[14] and FUCOUP[15] NMR measurements. These demonstrated that all of the original $^{13}$C-NMR assignments were correct but that the peaks due to C11 and C13 (75.3 and 75.9 ppm in DMSO-$d_6$, resp.) had changed relative positions (77.7 and 76.8 PPM in $CD_2Cl_2$, resp.)(table1). In some $^{13}$C-NMR spectral experiments signals due to C17 and C19 superimposed.

Initial biosynthetic studies commenced by pulse feeding ($^{13}CH_3$) methionine and Na(1-$^{13}$C)acetate to growing cultures, harvesting after 6 days and purifying the antibiotic by chromatography on C-18 bonded reverse phase silica gel. As expected, the O-methyl, C-23, was labeled strongly by methionine without any apparent change in the relative intensity of the signals of the other carbons. Consequently, the area of this signal was used as an internal standard to smooth out any variations in the NMR experiments and to calculate the degree of enhancement of various carbon atoms. Carboxyl labeled acetate gave at least a two-fold enhancement of all of

**Table 1 Assigned $^{13}$C-NMR Spectrum of Coloradocin in $CD_2Cl_2$[16]**

| Carbon | Signal | Carbon | Signal | Carbon | Signal | Carbon | Signal |
|---|---|---|---|---|---|---|---|
| 1 | 173.4 | 9 | 71.0 | 17 | 72.6 | 25 | 33.8 |
| 2 | 83.2 | 10 | 41.2 | 18 | 66.9 | 26 | 18.9 |
| 3 | 33.4 | 11 | 77.7 | 19 | 72.7 | 27 | 134.1 |
| 4 | 38.7 | 12 | 38.4 | 20 | 16.1 | 28 | 166.1 |
| 5 | 130.6 | 13 | 76.8 | 21 | 15.3 | 29 | 164.8 |
| 6 | 128.7 | 14 | 142.3 | 22 | 64.8 | 30 | 138.7 |
| 7 | 30.1 | 15 | 123.9 | 23 | 58.2 | 31 | 97.2 |
| 8 | 28.3 | 16 | 37.6 | 24 | 172.2 | 32 | 157.1 |

the expected carbons (1, 3, 5, 7, 11) in the upper tricyclic ring (based upon the nargenicin hypothesis), Table 2. Label was also found at a few of the carbons expected to be propionate derived (9, 13, 15, 17, 21) suggesting the presence of a hyperactive tricarboxylic acid cycle as has been noted in similar studies.[17] This was unfortunate, for the meaning of label appearing in C-24, 28, 29 and 32 in the lower bicyclic ring was clouded. The labeling pattern in carbons 24-32 was tail to tail rather than the head to tail pattern normal for polyketides. This could readily be rationalized by proposing that this unit is assembled after some TCA cycling as consequence of which succinate is constructed in this manner.

Complementary results were obtained from a feeding experiment with $^{13}$C-2 enriched acetate. Modest enrichment in C-26 suggested that the source of this carbon , for which, previously, we had no information was a primary metabolite strongly connected to acetate. Carbons suspected of originating in either the C-2 or the C-3 carbons of propionate were heavily enriched. To deal with this, an experiment was performed with Na(2-$^{13}$C)acetate in which a significant dose of unlabeled sodium propionate was added to suppress the activity of the tricarboxylic acid cycle and its attendant partial randomization of label. This experiment worked quite well in that randomization into labeled propionate was indeed suppressed. The label was now present in the upper ring system (enhancements greater than two-fold) only in the expected carbon locations for acetate origins (2, 4, 6, 8, 12). In the lower ring system, the suppression feeding experiment had a less profound influence. Enhancement found at C-25 was still strong, confirming an acetate origin for the C-24,25 unit but results for the rest of the ring system were less clear. C-26 and C-27 show modest enrichment but a similar level seen in C-31 is suppressed by propionate. It appears from this and additional experiments described below that the bicyclic unit is constructed and then added to the tricyclic unit later after a time when the TCA cycle has been in operation longer because incorporation in the bicyclic unit are nearly always less. While uncommon with fermentation products, it is not unprecedented. [18]

In order to confirm these findings to the extent possible and to determine securely the incorporation of intact acetate units, Na (1,2-$^{13}C_2$)acetate feeding was studied. Propionate suppression of randomization was also part of this experiment which had the added advantage of not only showing the degree of incorporation but when analyzed using a 2-D INADEQUATE experiment,[19] showed that the two-carbon acetate units were incorporated intact. In the upper ring system, as expected, units 1,2; 3,4; 5,6; 7,8 and 11,12 were labeled both in the propionate suppressed and the unsuppressed experiments. Propionate-derived carbons 9,10; 13,14; 15,16; and 17,18 were labeled to some extent in the experiment lacking propionate addition. The label in propionate derived carbons was incompletely but serviceably suppressed by this substrate. In the lower ring system, an acetate origin hypothesis for carbons 24,25 seems secure. Although acetate was implicated in the origin for the remaining carbons, C26 to 32, its route to incorporation was clearly indirect. In both 2-D INADEQUATE experiments connectivity within each pair of the expected acetate derived carbons including carbons 24 and 25 was clearly evident. Only in the experiment without propionate addition, was connectivity seen between the C1, C2 propionate derived carbons 9,10; 13,14; 15,16; 17,18 and between carbons 27,28; 29,30 and 31,32.

Feeding with Na (1-$^{13}$C)propionate lead to enrichment in the upper ring 5-7 fold at C-9, 13, and15. Unfortunately, the single unresolved peak which arose from both C-17 and C-19 made the C-17 results (not shown), although reasonable, less then definitive. Interestingly, the lower ring was labeled only at C-28 and C-29 by propionate (but with only two fold enrichment). This experiment largely confirmed a nargenicin/nodusmicin-like origin for the upper ring system and ruled out a propionate origin for C-26 to C-28, at least in the normal polyketide type orientation.

**Table 2 Effect of $^{13}C$ Enriched Acetate and Propionate Feeding Alone or in the Presence of Unenriched Propionate: Enrichment by Carbon Number**

| Carbon Number | [1-$^{13}C$] acetate | [2-$^{13}C$] acetate | [1,2-$^{13}C$] acetate + unenriched propionate | [1-$^{13}C$] propionate | [1,2-$^{13}C$] acetate[20] | [1,2-$^{13}C$] acetate + unenriched propionate |
|---|---|---|---|---|---|---|
| 1 | 4.1 | 1.0 | 1.3 | 1.0 | 2.7 | 3.8 |
| 2 | 1.0 | 4.5 | 4.3 | 1.2 | 2.5 | 4.6 |
| 3 | 4.5 | 1.0 | 1.1 | 1.1 | 2.2 | 4.7 |
| 4 | 1.1 | 4.2 | 3.7 | 1.0 | ** | 3.5 |
| 5 | 4.5 | 1.0 | 1.0 | 1.0 | 2.0 | 3.7 |
| 6 | 1.0 | 4.3 | 3.7 | 1.0 | 2.2 | 3.7 |
| 7 | 3.6 | 0.8 | 1.0 | 1.2 | 1.9 | 3.6 |
| 8 | 1.2 | 4.4 | 3.7 | 1.0 | 2.4 | 4.3 |
| 9 | 1.8 | 1.6 | 0.9 | 6.9 | 1.3 | 0.9 |
| 10 | 1.1 | 3.3 | 1.6 | 1.0 | 1.5 | 1.2 |
| 11 | 4.5 | 1.0 | 1.1 | 1.2 | 2.9 | 3.6 |
| 12 | 1.0 | 4.7 | 3.7 | 1.0 | ** | 3.5 |
| 13 | 1.7 | 1.0 | 0.8 | 5.7 | 1.7 | 0.9 |
| 14 | 0.9 | 2.3 | 1.2 | 1.0 | 1.3 | 1.0 |
| 15 | 2.7 | 1.5 | 1.0 | 5.8 | 1.3 | 1.1 |
| 16 | 1.3 | 2.7 | 1.1 | 1.1 | 1.6 | 1.1 |
| 17 | ** | ** | ** | ** | 1.3 | ** |
| 18 | 1.4 | 3.7 | 1.1 | 0.9 | 1.3 | 1.2 |
| 19 | ** | ** | ** | ** | 1.6 | ** |
| 20 | 1.0 | 3.3 | 1.1 | 1.2 | 1.3 | 1.2 |
| 21 | 1.0 | 2.4 | 1.0 | 0.9 | 1.3 | 1.2 |
| 22 | 1.4 | 2.6 | 1.0 | 1.1 | 1.3 | 1.0 |
| 23 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 24 | 4.2 | 1.0 | 1.9 | 0.8 | 2.9 | 3.1 |
| 25 | 1.3 | 4.3 | 3.3 | 0.8 | 3.1 | 3.1 |
| 26 | 1.2 | 2.4 | 1.7 | 1.0 | 1.2 | 1.4 |
| 27 | 1.0 | 1.3 | 1.9 | 1.2 | 1.6 | 1.6 |
| 28 | 1.9 | 1.4 | 1.2 | 2.1 | 1.5 | 1.7 |
| 29 | 2.5 | 0.9 | 0.9 | 2.0 | 1.6 | 1.4 |
| 30 | 0.9 | 1.4 | 1.1 | 1.0 | 1.7 | 1.1 |
| 31 | 1.1 | 2.0 | 1.2 | 1.2 | 1.5 | 1.5 |
| 32 | 3.9 | 1.0 | 0.9 | 0.9 | 1.8 | 1.3 |

** Indeterminate due to peak overlap

These studies left the origin of the lower ring system unclear. The data supported a direct or near direct acetate origin for C-24,25 and, at best, a very indirect acetate origin for C-26 to C-32 but with the acetate segments in the later unit attached tail to tail rather than in the polyketide, head to tail, fashion. Conversion of acetate to succinate could rationalize this finding satisfactorily.

In order to examine the putative role of succinate directly, feeding experiments were performed with sodium (2,3-$^{13}C_2$)succinate alone and in the presence of added unenriched sodium acetate and added unenriched sodium propionate. The results are shown in Table 3. Overall incorporation is relatively low and in the upper unit clear evidence was seen for scrambling of label through the tricarboxylic acid cycle. Carbons derived from C-2 and C-3 of propionate C-10,14,16, 18, 20, 21, and 22, all showed enrichment as would be expected by isomerization of succinate to methylmalonate. However slight elevations were seen in virtually all of the carbons in this section of the molecule. Suppression attempts with acetate were uniformly ineffective. In contrast, the effect of added propionate was quite definitive for carbons derived from this substrate. In the lower chain, the results were much more revealing. A near doubling of the abundance of label at carbons 26

**Table 3 Effect of Feeding [2,3-$^{13}$C] Succinate Alone or in the Presence of Unenriched Acetate and Unenriched Propionate : $^{13}$C Enrichment by Carbon Number.**

| Carbon Number | [2,3-$^{13}$C] succinate | [2,3-$^{13}$C] succinate + acetate | [2,3-$^{13}$C] succinate + propionate | Carbon Number | [2,3-$^{13}$C] succinate | [2,3-$^{13}$C] succinate +acetate | [2,3-$^{13}$C] succinate +propionate |
|---|---|---|---|---|---|---|---|
| 1 | 1.6 | 1.2 | 1.5 | 16 | 1.8 | 2.2 | 1.1 |
| 2 | 1.5 | 1.3 | 1.5 | 18 | 2.1 | 2.4 | 1.1 |
| 3 | 1.6 | 1.6 | 1.2 | 20 | 2.1 | 2.3 | 0.9 |
| 4 | 1.5 | 1.5 | 1.2 | 21 | 1.8 | 1.9 | 1.1 |
| 5 | 1.1 | 1.7 | 1.3 | 22 | 2.5 | 3.4 | 1.1 |
| 6 | 1.2 | 1.4 | 1.2 | 23 | **1.0** | **1.0** | **1.0** |
| 7 | 1.3 | 1.2 | 2.0 | 24 | 1.5 | 1.1 | 1.1 |
| 8 | 1.6 | 1.8 | 1.3 | 25 | 1.4 | 1.3 | 1.1 |
| 9 | 1.4 | 1.5 | 1.0 | 26 | 2.0 | 2.2 | 2.0 |
| 10 | 3.0 | 2.0 | 1.3 | 27 | 1.6 | 1.6 | 1.7 |
| 11 | 1.5 | 1.3 | 1.2 | 28 | 1.1 | 1.1 | 0.9 |
| 12 | 1.6 | 1.8 | 1.2 | 29 | 0.8 | 0.8 | 0.8 |
| 13 | 1.1 | 1.5 | 0.9 | 30 | 0.9 | 0.7 | 0.8 |
| 14 | 1.7 | 1.6 | 1.3 | 31 | 0.8 | 1.1 | 1.2 |
| 15 | 2.0 | 1.7 | 1.1 | 32 | 1.0 | 1.3 | 0.9 |

* Overlap of signals for C-17 and C-19 precluded an individual analysis for these two carbons

and 27, which was virtually unaffected by either acetate or propionate addition, provides direct evidence that these carbons (and C-28) come from succinate. This is further supported by a strong cross peak between these two carbon signals in the INADEQUATE experiment. It requires, however, that one of the carboxyl carbons be lost along the way. Surprising was the complete lack of enrichment in C-29-C32 segment, ruling out the suggestion from the acetate feeding experiments, that succinate was the source of these carbons. The experimental results indicate that C-2, C-3 of succinate find their way into the O-methyl (C-23) to a greater extent than to C-29 or C-30! One has to suppose that this C-29 to C-32 moiety is assembled indirectly form acetate by withdrawal from the TCA cycle, and that succinate cannot substitute in this process whereas C-26 to C-28 come from intact succinate with loss of one of the carboxyl carbons.

**Table 4 Ratio of 1-Bond Coupled Carbon to Uncoupled Carbons by Carbon Number in Coloradocin after feeding [U-$^{13}$C]-Glucose**

| Carbon | ΣC/U | Carbon | ΣC/U | Carbon | ΣC/U | Carbon | ΣC/U |
|---|---|---|---|---|---|---|---|
| 1 | 1.1 | 9 | 0.25 | 17 | * | 25 | 0.93 |
| 2 | 1.0 | 10 | 0.30 | 18 | 0.17 | 26 | 0.35 |
| 3 | 0.86 | 11 | 0.74 | 19 | * | 27 | 0.40 |
| 4 | 0.80 | 12 | 0.85 | 20 | 0.36 | 28 | 0.25 |
| 5 | 0.74 | 13 | 0.12 | 21 | 0.26 | 29 | <0.1 |
| 6 | 0.74 | 14 | 0.11 | 22 | 0.45 | 30 | <0.1 |
| 7 | 0.68 | 15 | 0.25 | 23 | <0.01 | 31 | 0.25 |
| 8 | 0.97 | 16 | 0.15 | 24 | 0.95 | 32 | 0.44 |

In a further attempt to throw some light into the origin of C-29 to C-32 an experiment was performed in which [U-$^{13}$C] glucose was fed. Given the central role of glucose in primary metabolism it did not seem reasonable to consider relative enrichment to an assumed standard (C-23). Rather we chose to examine the extent to which a particular carbon came from glucose as part of an intact two or three carbon fragment. This can be readily assessed by comparing the sum of the intensities of the coupled$^{13}$C signals to the uncoupled

signal.[21] These are shown in table 4.

These results indicate that, in the media used, glucose is metabolized to acetate and as such incorporated into coloradocin. The propionate units, and the succinate unit (C-26 to C-28) owe their origin to a lesser extent to two or three carbon fragments from glucose. In keeping with the earlier acetate incorporation experiments, C-31 and C-32 had some origin from glucose as a two carbon unit. In striking contrast was the complete lack of one bond carbon-carbon coupling seen for both C-29 and C-30. It is difficult to rationalize this result with the earlier experiments unless C-29 and C-30 are siphoned from the acetate pool early in the growth cycle into some as yet unidentified intermediate whose biosynthesis is essentially complete by the time the added glucose is metabolized to acetate. What is clear is that C-29 to C-32 are likely assembled by an uncommon pathway.

An attempt at incorporation of $^{13}$C-3-labeled oxaloacetate did not produce sufficient incorporation to be interpeted nor did one with $^{13}$C-1-labeled glutamate. These experiments rule out the possibility of a biosynthetic pathway similar to that of the nonandrides[13].

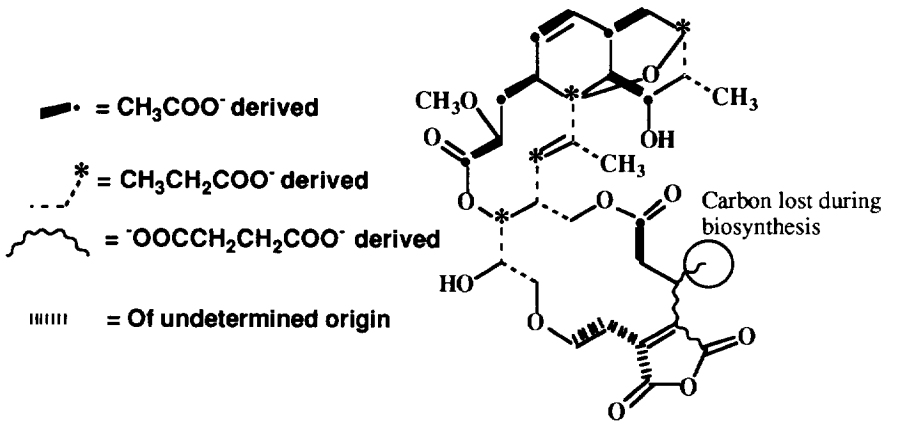
**Table 5 Ratio of 1-Bond Coupled Carbon to Uncoupled Carbons by Carbon Number in Coloradocin after feeding [U-$^{13}$C]-Glucose and unenriched Sodium Acetate**

| Carbon | ΣC/U | Carbon | ΣC/U | Carbon | ΣC/U | Carbon | ΣC/U |
|---|---|---|---|---|---|---|---|
| 1 | 1.2 | 9 | 0.29 | 17 | * | 25 | 1.0 |
| 2 | 1.2 | 10 | 0.33 | 18 | 0.33 | 26 | 0.36 |
| 3 | 0.64 | 11 | 0.95 | 19 | * | 27 | 0.42 |
| 4 | 1.4 | 12 | 1.4 | 20 | 0.40 | 28 | 0.48 |
| 5 | 0.93 | 13 | 0.29 | 21 | 0.36 | 29 | 0.60 |
| 6 | 1.0 | 14 | 0.23 | 22 | 0.42 | 30 | 0.67 |
| 7 | 0.89 | 15 | 0.28 | 23 | <0.05 | 31 | 0.64 |
| 8 | 1.1 | 16 | 0.31 | 24 | 1.1 | 32 | 0.86 |

## *CONCLUSION*

These studies demonstrated the presence of a hyperactive tricarboxyilc acid cycle which complicated interpretation of the effects of feeding simple labeled precursors. Loading with unlabeled propionate effectively suppressed label scrambling from both acetate and succinate. The findings confirm unambiguously the inference that the upper tricyclic ring system is a typical polyketide whose biosynthesis generally follows the pattern established for nodusmicin and nargenicin. The origin of the carbons, and in particular the path by which they are assembled, of the more unique lower bicyclic ring system was much more difficult to establish. The degree of incorporation of precursors was usually lower than in the upper tricyclic ring system, with the notable exception of carbons 24 and 25, suggesting either a larger pool, greater processing through the TCA cycle or biosynthesis at different time than the upper chain. The incorporation of acetate into carbons 24and 25 is at levels very similar to its incorporation in the polyketide chain. Succinate appears to be the origin for carbons 26-28. This requires that a carboxyl originally attached to C-26 was lost somewhere in the sequence. The evidence indicates that acetate units can feed into C29-32 but probably via circuitous routes and clearly not from succinate. The manner of attachment of C27 to C30 is not revealed by these experiments. It is very clear however that this lower ring portion is biosynthesized by pathways different from those few demonstrated for naturally occurring anhydrides.[10,11,12,13] Based upon these experiments, we believe that coloradocin is assembled from an upper polyketide chain and the lower portion receives strategic input from the TCA cycle in an as yet undefined pathway. A recently described metabolite from a *Penicillium* sp. has a carbon chain very similar to this section of coloradocin and may provide a useful model for further studies.[22]

**Figure Biosynthetic Precursors of Coloradocin**




COLORADOCIN

## *EXPERIMENTAL*

NMR spectra were measured on either a General Electric GN 300 or GN 500 spectrometer using 5mm probes and $CD_2Cl_2$ as solvent and internal reference taken as 53.8 ppm downfield from TMS.

### Fermentations

An initial seed culture of *Actinoplanes coloradoensis* AB 921J-26 was grown in 25 x 150 mL culture tubes containing 10 mL media consisting of starch 2.4%, yeast extract 1%, glucose 1%, Tryptone®0.5%, beef extract 0.3%, and $CaCO_3$ 0.4% prepared in distilled water and adjusted to pH7 before sterilization. Inoculation was at 5% from vegetative frozen seed which had been stored at -75°C and growth was on a rotary shaker at 30°C and 250 rpm with 3.2 cm stroke for 96 h. This was used to inoculate a second stage seed in 500 mL Erlenmeyer flasks containing 100 mL of the same medium which was similarly grown and used after 72 h to inoculate the fermentation medium. This consisted of glucose monohydrate 1%, starch 0.4%, molasses 0.5%, Lexein F-152 liquid peptone 0.4%, soybean flour 0.5%, NZ amine type A 0.1%, yeast extract 0.1%, $MgSO_4$ 0.02%, and $CaCO_3$ 0.1% at 100 mL per 500 mL Erlenmeyer flask. The rotary shaker had a 5.6 cm stroke. Conditions of growth were otherwise the same as the second stage seed and harvest was on the 6th d.

As a prelude to precursor studies the effects of several likely substrates on both growth of *A. coloradoensis* and the production of coloradicin were assessed in shaken flask fermentations. Sodium acetate, propionate, succinate and oxaloacetate were added to the fermentation at levels of from 1 to 6 g/L, bolus at time zero and on d3 and also in daily pulses. Acetate was tolerated at levels of up to 4g/L bolus or 8g/L in 2g/L a day pulses without adverse effects on growth or yield. Although bolus doses as low as 1g/L of propionate inhibited growth to some extent, coloradocin yields were higher at 2g/L bolus /d. If addition was delayed until d3 the organism could tolerate 4g/L without detriment to yield. Pulsing addition of propionate from d3 allowed total feeding of 7g/L of propionate . Although succinate was not detrimental to growth it had adverse effects on yields at levels as low as 3g/L. Oxaloacetate could be tolerated to levels of 3g/L but not at 6g/L and the study showed little difference whether it was added initially or at d3.

In precursor studies $^{13}C$ enriched substrates were added on each of d3, 4, and 5 at 0.01% or 0.02% with the exception of L-glutamic acid-1-$^{13}C$ and oxaloacetic acid-3-$^{13}C$ which were added at 0.003%. Natural abundance suppression agents were added on d1, 2, 3, and 4 at 0.1% /d for sodium acetate and 0.175% /d for sodium propionate.

### Isolation of coloradocin

At harvest the beer from 100 flasks was pooled, the pH was adjusted to 4 with sulfuric acid and

Amberlite XAD-2 resin (500g) was added and stirred. The resin and mycelia were filtered off, washed well with water and eluted with methanol (5 L). XAD-2 resin (15g) was added to the methanol and the mixture was concentrated to dryness. The XAD-2 resin, with adsorbed residue, was loaded onto a column (5cm x 40cm) of XAD-2 resin and this was eluted with a step gradient of water to methanol. Fractions containing coloradocin were combined and concentrated and the crude product was purified by chromatography on a low pressure column (2.5cm x 30cm) of C-18 RP silica gel eluted with $CH_3CN:H_2O$, 1:1 to obtain spectroscopically pure material.

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