J|A|C|S
ARTICLES
Published on Web 05/22/2002

Biosynthesis of Nodulisporic Acid A: Precursor Studies

Kevin M. Byrne,* Scott K. Smith, and John G. Ondeyka

Contribution from the Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065-0900

Received September 27, 2001

Abstract: Nodulisporic acid A (NAA) is an indole-diterpene natural product produced by an indeterminate species of the endophytic fungus *Nodulisporium*. NAA (Figure 1) is structurally related to the paspaline class of fungal metabolites. The biosynthetic origin proposed for these alkaloids involves the acetate/mevalonic acid pathway leading to geranylgeranyl pyrophosphate (GGPP). GGPP is then proposed to condense with tryptophan to form the basic indole-diterpene core. A washed cell procedure was devised to incorporate labeled precursors into NAA by a mutant *Nodulisporium* culture designated MF6244. Incorporation of 2-^{13}C-acetate and 2-^{13}C-mevalonolactone into NAA was found to occur in the classical mevalonic acid pattern. In addition to the four mevalonic acid units that form the eastern side of the molecule, three additional isoprenylations occur to form the western and southern regions of NAA. Contrary to published reports on related compounds, incubations of *Nodulisporium* MF6244 with ^{14}C- and ^{13}C-tryptophan showed no incorporation of label into NAA. However, high levels of incorporation into NAA were obtained with known tryptophan precursors ^{14}C-, ^{13}C-, and ^{15}N-anthranilic acid and ^{14}C- and ^{13}C-ribose. A novel pathway for the biosynthesis of NAA is presented.

Introduction

Nodulisporic acid A (NAA) is an indole-diterpene metabolite produced by *Nodulisporium* sp. (MF5954, ATCC 74245), an endophytic fungus isolated from a woody plant. NAA exhibits potent insecticidal activities against the mosquito larvae of *Aedes aegypti*, larvae of the blowfly *Lucilia seracata*,[1] and the common cat flea *Ctenocephalides felis*.[2] The mechanism of action of NAA appears to be similar to that of ivermectin, but NAA acts through a more selective set of insect, glutamate-gated chloride channels.[3] NAA (Figure 1) is structurally related to the paxilline class of fungal metabolites which includes paspaline/paspalicine,[4] paxilline,[5] the penitrems,[6] the janthitrems,[7] the lolitrems,[8,9] and the shearinines.[10] However, several chemical features of NAA make it a unique member of this indole-diterpene class of alkaloids. NAA lacks the tertiary hydroxyl group at C-9 that is implicated in the tremorgenic properties of the related alkaloids. In addition, NAA contains a unique cyclopentyl ring in the western region and a highly constrained β-keto-dihydropyrrole ring in the southern region of the molecule. A very limited number of radioactive and stable isotope experiments have been reported on this class of alkaloids. Establishment of culture conditions capable of supporting production levels required for incorporation studies has been problematic for many of the indole-diterpene producers.[11,12] In the case of the parent *Nodulisporium sp.* MF5954, medium studies enabled the development of submerged fermentation conditions that supported production levels higher than 10 mg/L. Subsequent strain

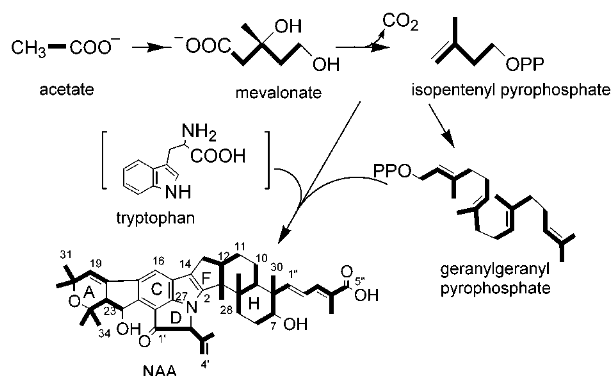

Figure 1. A pathway was proposed for the biosynthesis of NAA on the basis of previously reported labeling studies for the biosynthesis of paspaline[13] and penitrem A.[14] Bold lines indicate an IPP-derived unit.

* To whom correspondence should be addressed. E-mail: kevin_byrne@merck.com.

(1) Ondeyka, J. G.; Helms, G. L.; Hensens, O. D.; Goetz, M. A.; Zink, D. L.; Tsipouras, A.; Shoop, W. L.; Slayton, L.; Dombrowski, A. W.; Polishook, J. D.; Ostlind, D. A.; Tsou, N. N.; Ball, R. G.; Singh, S. B. *J. Am. Chem. Soc.* **1997**, *119*, 8809−8816.
(2) Shoop, W. L.; Gregory, L. M.; Zakson-Aiken, M.; Michael, B. F.; Haines, H. W.; Ondeyka, J. G.; Meinke, P. T.; Schmatz, D. M. *J. Parasitol.* **2001**, *87*, 419−423.
(3) Smith, M. M.; Warren, V. A.; Thomas, B. S.; Brochu, R. M.; Ertel, E. A.; Rohrer, S.; Schaeffer, J.; Schmatz, D.; Petuch, B. R.; Tang, Y. S.; Meinke, P. T.; Kaczorowski, G. J.; Cohen, C. *J. Biochem.* **2000**, *39*, 5543−5554.
(4) Springer, J. P.; Clardy, J. *Tetrahedon Lett.* **1980**, *21*, 231−234.
(5) Springer, J. P.; Clardt, J.; Wells, J. M.; Cole, R. J.; Kirksey, J. W. *Tetrahedon Lett.* **1975**, *30*, 2531−2534.
(6) de Jesus, A. E.; Steyn, P. S.; Van Heerden, F. R.; Vleggaar, R.; Wessels, P. L.; Hull, W. E. *J. Chem. Soc., Chem. Commun.* **1981**, 289−298.
(7) de Jesus, A. E.; Steyn, P. S.; Van Heerden, F. R.; Vleggaar, R. J. *J. Chem. Soc., Perkin Trans. 1* **1984**, 697−701.
(8) Gallagher, R. T.; Hawkes, J. D.; Van Heerden, F. R.; Vleggaar, R. J. *J. Chem. Soc., Chem. Commun.* **1984**, 614−616.
(9) Munday-Finch, S. C.; Wilkens, A. L.; Miles, C. O.; Yomoda, H.; Omura, S. *J. Agric. Food Chem.* **1995**, *43*, 1283−1288.
(10) Belofsky, G. N.; Gloer, J. B. *Tetrahedron* **1995**, *51*, 3959−3968.
(11) Laws, I.; Mantle P. G. *J. Gen. Microbiol.* **1989**, *135*, 2679−2692.
(12) Penn, J.; Mantle, P. G. *Phytochemistry* **1994**, *35*, 921−926.

Table 1. Summary of ^{13}C Enrichments and Coupling Constants Resulting from Incorporation of ^{13}C-Labeled Precursors into NAA

carbon	^{13}C δ,ppm	2-^{13}C-acetate[a]	1,2-$^{13}C_2$-acetate[a]	1,2-$^{13}C_2$-acetate {$J_{13C-13C}$(Hz)}
2	155.7		0	0
3	56.1		2.76	36.1[c]
4	39.1		2.77	36.8[c]
5	32.3	5	3.05	(33.0[c])
6	25.95		3.29	35.8[c]
7	76.8	3	3.35^{3}	37.4[c]
8	47.8		solv	38.8[c]
9	45.2	3	3.27	32.1[c]
10	24.7		3.56	35.1[c]
11	25.7	3 (34.0)[b]	3.29	(32.2[c])
12	48.0	3 (34.0)[b]	solv	solv
13	27.7		3.01	33.0
14	122.7		0	0
15	121.8		0	0
16	116.7		0	0
17	134.0		0.90	0
18	135.9		1.93	73.9[c]
19	122.0	4	0.90	73.2[c]
20	72.6		2.41^{2}	40.3[d]
22	73.9		2.41^{2}	36.5[d]
23	58.2	3	1.66	36.6[c]
24	75.3		2.41^{2}	40.0
25	138.4		0	0
26	113.1		0	0
27	163.2		0	0
28	15.1	4	3.04	36.0
29	19.6	4	3.05	36.6
30	11.2	3	3.19	36.6
31	29.9	2	2.51	(41.0)
32	31.9	3	3.20	41.0
33	23.4	3	2.38	(38.8)
34	30.1	4	2.51	38.8
1′	198.0		2.39	39.6
2′	76.4	3	3.53^{3}	39.0[c]
3′	140.0		3.59^{5}	74.0[c]
4′	117.5		1.64	70.0[c]
5′	18.1	3	4.10	(42.0)
1″	154.6	4	2.14	(41.8[c])
2″	125.9		3.38^{4}	54.0[c]
3″	140.8	3	3.59^{5}	58.2[c]
4″	125.1		3.38^{4}	46.2[c]
5″	172.6	3	3.74	(78.0)
6″	12.6	3	3.52	44.8

[a] Values indicate X-fold enrichment levels as compared with natural abundance. [b] Spectrum taken in acetone-d_6 to observe the C-12 region. X^n $n = 2, 3, 4$, or 5, where two or more carbons are integrated together. [c] Additional splitting is present. [d] Satellites associated with C-20 and C-22 were broad and slightly distorted owing to similar coupling constants arising from the one intraacetate pair (i.e., C-20/C-32 and C-22/C-34) overlaid with the interacetate coupling (i.e., C-20/C-31 and C-20/C-33). Values in parentheses result from multiple labeling.

improvements (MF6244 and MF6299) resulted in further increases in product titers making stable isotope studies possible. On the basis of previous studies of the incorporation of ^{14}C- and ^{13}C-labeled precursors into paspaline and penitrem A by *Claviceps paspali* and *Penicillium crustosum*, respectively, it was proposed that the indole core and eastern region of this class of alkaloids arise by condensation of tryptophan or a tryptophan metabolite with an activated diterpene, presumably geranylgeranyl pyrophosphate (GGPP).[13,14] The involvement of GGPP was made even more likely with the recent publication describing the cloning of the first indole-diterpene gene cluster from the paxilline producing fungus *Penicillium paxilli*.[15] A gene for GGPP, paxG, was identified within the gene cluster which

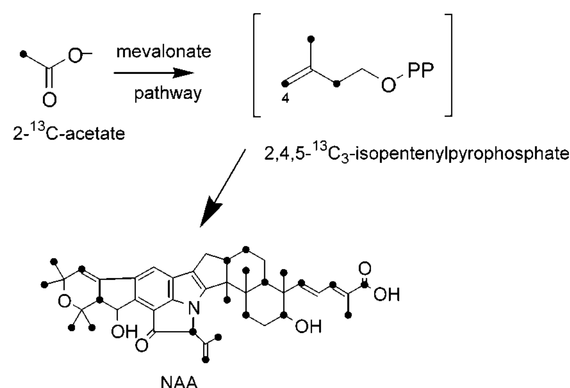

Figure 2. Incorporation of 2-^{13}C-acetate into NAA demonstrated the involvement of the classic acetate/mevalonate pathway in the biosynthesis of NAA. The labeling pattern is consistent with the incorporation of seven isoprene units into NAA. The small darkened circle • indicates sites of enrichment.

was essential for paxilline biosynthesis. The studies reported here were undertaken to investigate if the biosynthesis of NAA by *Nodulisporium sp.* (MF6244 and MF6299) used such a mevalonate-tryptophan biosynthetic pathway (Figure 1), and to elucidate the origin of the western and southern regions unique to NAA.

Acetate. Metabolism of 2-^{13}C-acetate through the classical mevalonic acid pathway leads to enrichment of carbons 2, 4, and 5 of isopentenyl pyrophosphate (IPP).[16] Supplementation of washed cells of MF6244 with 2-^{13}C-acetate resulted in NAA enrichment that confirmed biosynthesis via the mevalonic acid pathway (Table 1). The enrichments at carbons 5, 7, 9, 11, 19, 23, 28, 29, 30, 31, 32, 33, 34, 2′, 5′, 1″, 3″, 5″, and 6″ were consistent with the model that 2-^{13}C-acetate was incorporated into four units of 2,4,5-$^{13}C_3$-IPP, which then condensed to form the geranylgeranyl pyrophosphate-derived eastern end of NAA (Figure 2). The labeling pattern was also consistent with three additional isoprenylations to form the western and southern regions.

The only carbon resonance exhibiting evidence of coupling in this spectrum taken in CD_3OD was C-11. The ^{13}C NMR spectrum of this same enriched NAA sample obtained in acetone-d_6 revealed the second coupled carbon at 48 ppm (masked in CD_3OD) with a coupling constant of 34.0 Hz (Figure 3). The magnitude and size of the coupling between C-11 and C-12 were evidence that a rearrangement had occurred during the condensation and cyclization of GGPP with the indole precursor, resulting in proximate bonding between these two C-2-acetate-derived carbon atoms. We propose a 1,2-bond shift rearrangement as depicted in Figure 3. These data are consistent with a similar rearrangement proposed for the cyclization of paspaline[13] and penitrem A.[14] Incorporation of 1,2-$^{13}C_2$-acetate into NAA resulted in prominent coupling between those carbon atoms that arose from intact acetate units (Table 1). It is well established that three acetate units are utilized in the biosynthesis of each mevalonic acid precursor molecule.[16] While two acetate units remain intact, a loss of C-1 of mevalonic acid as CO_2 must occur in conversion to IPP. This results in an uncoupled carbon of C-2 acetate origin at C-4 of IPP. Consequently, 12

(13) Acklin, W.; Weibel, F.; Arigoni, D. *Chimia* **1977**, *31*, 63.
(14) de Jesus, A. E.; Steyn, P. S.; Van Heerden, F. R.; Vleggaar, R. J.; Wessels, P. L. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1863−1868.
(15) Young, C.; McMillan, L.; Telfer, D.; Scott, B. *Mol. Microbiol.* **2001**, *39*, 754−764.
(16) Eisenreich, W.; Schwarz, M.; Cartayrade, A.; Arigoni, D.; Zenk, M. H.; Bacher, A. *Chem. Biol.* **1998**, *5*, R221−R233.

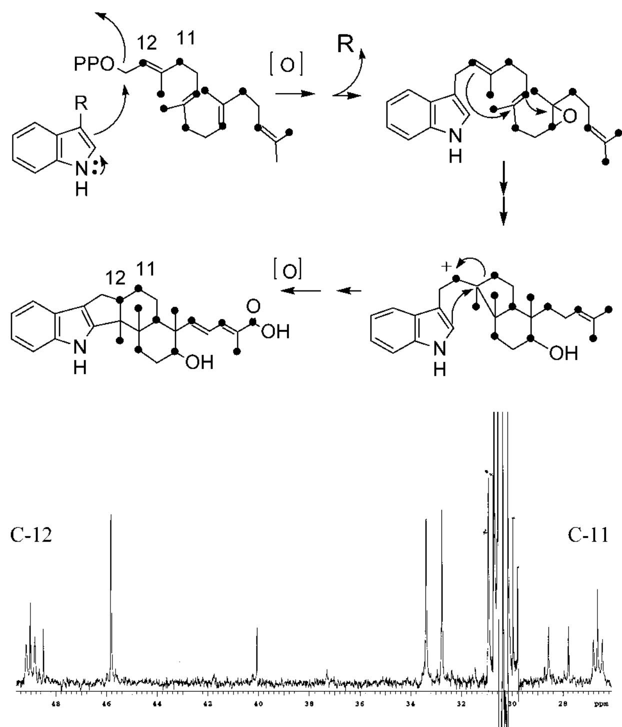

Figure 3. A prominent coupling of 34.0 Hz was observed between C-11 and C-12 of NAA produced in the presence of 2-^{13}C-acetate. The rearrangement of C-11 proximate to C-12 suggests the occurrence of a 1,2-bond shift in the condensation and cyclization of GGPP with the indole precursor in NAA biosynthesis. Aklin and de Jesus proposed a similar 1,2-bond shift to explain the formation of ring F in paspaline[13] and penitrem A,[14] respectively. Spectrum obtained in acetone-d_6. R is the side chain of the indole intermediate as proposed in Figure 5.

acetates (eight coupled) were utilized for production of the geranylgeranyl pyrophosphate that went on to form the eastern side of NAA. Nine acetates (six coupled) gave rise to the three IPPs that formed the western and southern regions.

Experimentally, coupling constants of 70.0−74.0 Hz were observed for the olefinic linkages between C-3′/C-4′ and C-18/C-19. A coupling of 54−58.2 Hz was observed for the intact acetate unit (C-3″/C-2″) contained in the conjugated diene-carboxylic acid side chain. The C-6″ methyl substituent on the same side chain was coupled to C-4″ with a coupling of 44.8−46.2 Hz. The C-12 coupling at 48.0 ppm arising from the acetate precursor at C-11/C-12 fell under the CD_3OD solvent peak. However, the 33.0 Hz coupling of C-12 to C-11 was clearly observable as satellites on the C-11 resonance at 25.7 ppm. All of the remaining 10 coupled acetate pairs possessed similar coupling constants in the range from 32.1 to 41.0 Hz. In addition, the high level of incorporation of 1,2-$^{13}C_2$-acetate led to interacetate couplings between NAA carbons derived from carbons 3 and 4 of IPP. For example, C-31 arises from C-2 of acetate and C-4 of IPP after loss of its contiguous C-1 acetate carbon as CO_2 (Figure 1). However, a coupling of 41.0 Hz was observed at 29.9 ppm because the high level of acetate incorporation into NAA led to interacetate coupling between C-31 and C-20. This was observed for all of the C-4 IPP-derived carbons of NAA.

Mevalonic Acid. 2-^{13}C-Mevalonolactone would be expected to lead to 4-^{13}C-IPP in isoprenoid biosynthesis. Supplementing washed cells of MF6244 with 2-^{13}C-mevalonolactone confirmed both the acetate labeling pattern shown above and the involvement of the mevalonic acid pathway (Table 1). Extremely high levels of enrichment (35−45-fold) were observed at carbons 5″, 1″, 5, 11, 5′, 31, and 33, one site of enrichment for each isoprenoid unit incorporated. The level of enrichment into NAA and the lack of significant labeling at other sites in NAA demonstrated that mevalonolactone was incorporated directly into isopentenyl pyrophosphate without scrambling of the label through competitive metabolic pathways.

Table 2. Incorporation of (Ring-$^{14}C_6$)-anthranilic Acid[a] into NAA and Hinnuliquinone

AA (nM)	L-Trp (mM)	DPM into hinnuliquinone	DPM into NAA	% incorporation into NAA
0.06	0	78 500	25 800	1.63
0.16	0	82 400	23 800	1.59
1.06	0	45 800	16 000	1.05
10.06	0	13 200	0	0
0.16	1.0	22 800	40 000	2.67
0.16	10.0	0	55 400	4.2

[a] Addition of 0.7 μCi was made to 1.0 mL of washed cells prepared at 6 days and incubated in 17 × 100 mm tubes for 72 h. Analysis was performed on 1.0 mL of whole broth.

Tryptophan versus Anthranilic Acid. All attempts to label NAA with indole-^{14}C-tryptophan were unsuccessful, as were efforts to achieve ^{13}C incorporation from either 2-^{13}C-indole-tryptophan or 2-^{13}C-indole. These results were unexpected in view of the reported incorporation of tryptophan into the indole-diterpenes penitrem A and paxilline.[11,14,17] Evidence for the involvement of a precursor earlier than tryptophan in the classic, aromatic amino acid biosynthetic pathway was obtained by supplementing washed cells of MF6244 with (ring-$^{14}C_6$)-anthranilic acid. Anthranilic acid (AA) incorporation into NAA alone was 1.6%, while greater than 6% of the added (ring-$^{14}C_6$)-anthranilic acid was incorporated into the entire family of NAA compounds (Table 2). Results of a competition experiment between L-tryptophan and (ring-$^{14}C_6$)-anthranilic acid in MF6244 cells are also shown in Table 2. The pigment hinnuliquinone (see Supporting Information for structure), a known tryptophan-derived co-metabolite of NAA produced by this culture, was used as an internal reference compound.[18] Instead of unlabeled tryptophan diluting the labeled anthranilic acid incorporated into NAA, as expected if tryptophan was a precursor, and as occurred for hinnuliquinone, addition of unlabeled L-tryptophan led to a 2.5-fold increase in incorporation into NAA. These data provided strong evidence that tryptophan was not a precursor in the pathway from anthranilic acid to NAA. It also explained the increase in incorporation of anthranilic acid into NAA because the presence of excess tryptophan in the medium would be expected to down-regulate synthesis of tryptophan, allowing for increased shunting of anthranilic acid into NAA.

If anthranilic acid was incorporated into NAA by MF6244, using a pathway analogous to tryptophan biosynthesis, the carboxyl group of anthranilic acid would be lost as CO_2 and not incorporated into NAA. A comparison of incorporation into NAA of (ring-$^{14}C_6$)-anthranilic acid and (carboxyl-^{14}C)-anthranilic acid was done in washed cells. Forty-eight hour incubations resulted in a 2.0% incorporation of 0.6 mM (ring-$^{14}C_6$)-anthranilic acid into NAA, while no incorporation was obtained from 0.6 mM (carboxyl-^{14}C)-anthranilic acid.

(17) Mantle, P. G.; Weedon, C. M. *Phytochemistry* **1994**, *36*, 1209−1217.
(18) O'Leary, M. A.; Hanson, J. R.; Yeoh, B. L. *J. Chem. Soc., Perkin Trans. 1* **1984**, 567−569.

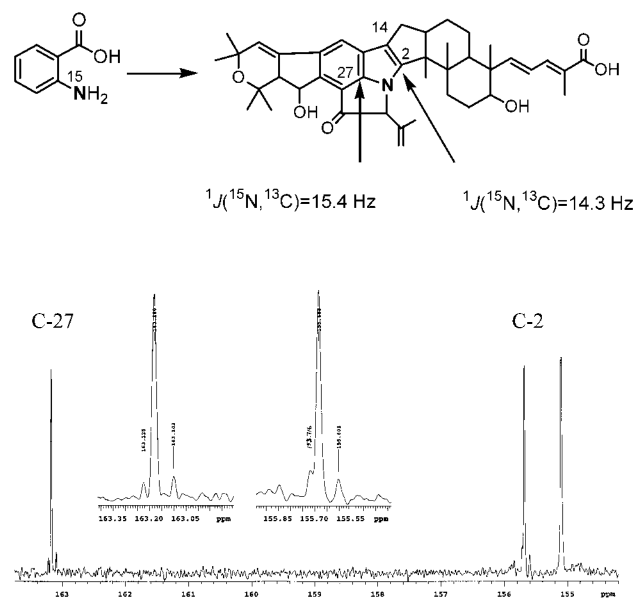

Figure 4. Incorporation of ^{15}N-anthranilic acid into NAA resulted in coupling of ^{15}N to the natural abundance ^{13}C nuclei at C-2 and C-27.

Supplementation of washed cells with both (ring-$^{13}C_6$)-anthranilic acid and ^{15}N-anthranilic acid was used to confirm the origin of the six benzene indole carbon atoms of NAA and the one nitrogen atom. All six benzene carbons of NAA were labeled from (ring-$^{13}C_6$)-anthranilic acid at an enrichment level of 2- to 3-fold (see Supporting Information). In addition, the six carbon signals were split into doublets-of-doublets, indicating that the ring carbon atoms were incorporated intact into NAA. Analysis of the carbon NMR spectrum of NAA produced in the presence of ^{15}N-anthranilic acid showed only two signals with distinct satellites resulting from ^{13}C-^{15}N coupling at C-2 and C-27 (Figure 4). The resonances at 155.7 and 163.2 ppm each contained a pair of satellites with couplings of 14.3 and 15.4 Hz, respectively, within the expected range of 15 Hz for ^{13}C/^{15}N coupling.

Ribose. If biosynthesis of the indole nucleus of NAA does occur by a pathway analogous to tryptophan biosynthesis, but diverges somewhere between phosphoribosylanthranilate and tryptophan, the last two carbons of the pyrrole ring of the indole should arise from carbons 1 and 2 of phosphoribosylpyrophosphate (PRPP). With the hope that a sufficient quantity of exogenously added ribose might be converted to PRPP and incorporated into NAA, MF6299 cells were supplemented at 8 days with either 1-^{13}C-D-ribose or [UL-$^{13}C_5$]-D-ribose. Because earlier studies had indicated that ribose can serve as a sole carbon source for growth of MF6244, considerable enrichment because of scrambling of label from ribose catabolism was expected. However, adding the labeled ribose to the cells toward the end of the growth phase eliminated much of the scrambling. The subsequent ^{13}C NMR spectrum of NAA produced in the presence of 1-^{13}C-D-ribose (see Supporting Information) exhibited a very distinct 2-fold enrichment at carbon 2 of NAA, the expected site of enrichment if the indole is formed by condensation of anthranilic acid and PRPP.

Additional evidence in support of ribose as the precursor of carbons 2 and 14 of NAA was obtained by supplementing resting cells of MF6299 with [UL-$^{13}C_5$]-D-ribose. Minor satellites were observed (10−40%) in a pattern similar to that

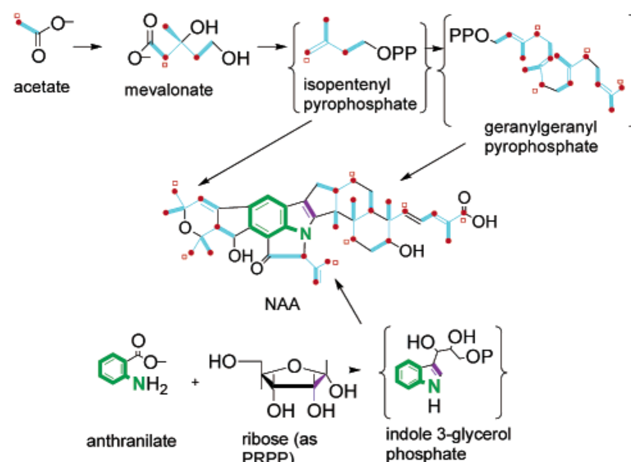

Figure 5. Summary of the biosynthetic scheme proposed for NAA.

obtained from 1,2-$^{13}C_2$-acetate incorporation into NAA. However, only C-2 and C-14 showed significant satellites (see Supporting Information) corresponding to a 2-fold enrichment with a coupling constant of 52 Hz. In addition to confirming the 1-^{13}C-D-ribose labeling, the coupling of C-2 to C-14 demonstrated that these carbon atoms arise intact from two adjacent carbons of ribose. The most probable explanation for this is if C-14 of NAA arises from C-2 of ribose. By comparison with known aromatic amino acid biosynthesis, phosphoribosylanthranilate would then go on to form indole-3-glycerol phosphate. Because this activated compound is the last intermediate in the pathway before the action of tryptophan synthase, it is likely the activated indole molecule that condenses with GGPP to form the indole-diterpene core.

Discussion

The purpose of the work described above was to establish the primary biosynthetic precursors involved in the biosynthesis of NAA. Labeling of NAA with ^{13}C- acetate and ^{13}C-mevalonate demonstrated that the isoprenoid region on the eastern side of NAA is derived from mevalonic acid through the classic acetate/mevalonic acid pathway, in agreement with the labeling patterns proposed for the penitrem class of indole-diterpene alkaloids.[6,13,14] This 20-carbon region of NAA arises from the condensation of four isoprene units followed by condensation of the diterpene with an indole nucleus (Figure 5). The hypothesis that the four isoprenes pass through a GGPP intermediate is supported by the sites of 2-^{13}C-mevalonolactone incorporation into NAA. The condensation of each isoprene unit through C-4 of each IPP was demonstrated by the specific incorporation of 2-^{13}C-mevalonolactone into carbons 1″, 5, and 11 of NAA. These three carbons correspond to the three isoprene condensation sites of GGPP biosynthesis from one unit of dimethylallyl pyrophosphate and three units of IPP. In contrast to the 20-carbon isoprenoid eastern region of NAA, the equivalent regions of penitrem A and paxilline contain 19 carbons. One methyl carbon, arising from a C-2 of acetate and a C-5 of IPP, is lost in formation of these two indole-diterpenes.[6,13]

During condensation of GGPP with the indole intermediate of penitrem A and paxilline, a 1,2-bond migration occurs at C-20.[14] The ^{13}C NMR spectrum of NAA derived from 2-^{13}C-acetate exhibited coupling that is consistent with the same

rearrangement occurring in NAA biosynthesis. The only prominent coupling exhibited in the spectrum was a 34 Hz coupling between C-11 and C-12 (Figure 3). Because both of these carbons are derived from C-2 of acetate, it necessitates the occurrence of a one-bond migration in the GGPP intermediate, most probably during the condensation and cyclization steps of GGPP with the indole intermediate. In the process, the bond between C-3 and C-4 of GGPP is cleaved, and C-4 bonds directly to the C-2-acetate-derived C-2 of GGPP. Carbon 3 of GGPP goes on to become C-3 of NAA, while C-2 and C-4 of GGPP go on to become C-12 and C-11 of NAA, respectively (Figure 3).

Three additional isoprenylations occur in NAA biosynthesis on three contiguous carbons in the indole nucleus. The 10-carbon skeleton that comprises rings A and B was enriched by ^{13}C-acetate and ^{13}C-mevalonate in patterns consistent with the attachment of two isoprene units at C-17 and C-25 of NAA. Attachment of these isoprenes is different in both site and mechanism than that proposed for penitrem A,[6] but occurs at the same position as in the case of the janthitrem mycotoxins.[7] However, cyclization of NAA to give rings A and B proceeds in an opposite fashion from the cyclization to give rings A and B of the janthitrems. The third isoprenylation occurs at C-26 to give rise to ring D and constitutes the southern region of the molecule. Incorporation of ^{13}C-labeled acetate and mevalonate confirmed the isoprene origin of this five-carbon attachment. The nodulisporic acids are the only members of this indole-diterpene class of alkaloids to possess this southern ring region.

While the isoprenoid portion of this class of indole-diterpene is quite readily labeled with either ^{14}C or ^{13}C mevalonate,[11−14] evidence supporting the hypothesis of de Jesus[14] that tryptophan serves as a precursor for the indole nucleus is problematic. Incorporation of only 0.16% benzene ring-[UL-^{14}C]-DL-tryptophan into penitrem A by a culture of *P. crustosum*[14] led the authors to propose that tryptophan was a metabolic precursor. The low incorporation rate was symptomatic of a major difficulty in working with this particular microorganism. Penitrem A is produced primarily by the spores of *P. crustosum* in stationary culture, necessitating the addition of the labeled precursor in solution form deposited onto the sporulating surface. This process is likely to lead to a gradient distribution of the precursors to the penitrem producing spores wherein the dynamics of the gradient process will be in part dependent on the hydrophobic properties of the particular precursor tested. A subsequent, more thorough, study was reported of tryptophan incorporation into penitrem A using *Penicillium crustosum*, and into the structurally similar metabolites hyroxymethylbutenyl-paspalinine by *Claviceps paspali* and paxilline by *Penicillium paxilli*.[11] The latter two labeling experiments involved submerged fermentations, obviating the gradient difficulties associated with the stationary culture conditions for penitrem A biosynthesis. These authors reported benzene ring-[UL-^{14}C]-DL-tryptophan incorporations of 3.6% into penitrem A, 2.6% into hyroxymethylbutenyl-paspalinine, and 5% into paxilline. While higher than the initial incorporation level of 0.16% for penitrem A, the levels are much lower than the 20−23% incorporation of ^{14}C-DL-tryptophan incorporated into roquefortine, a tryptophan-derived metabolite coproduced by *P. crustosum* under identical fermentation conditions.[11,19] Although these results lend more credence to the hypothesis that tryptophan can serve as a precursor for the indole nucleus, neither these nor subsequent reports included data on benzene ring-^{13}C-tryptophan labeling for this class of alkaloids.[11,19] Thus, no sites of incorporation were established, and the extent of labeling from metabolic scrambling remains uncertain.

When washed cells of MA6244 were incubated with [UL-^{14}C]-DL-tryptophan, no evidence of incorporation into NAA was obtained. Similarly, there was no indication of incorporation of L-tryptophan (indole-2-^{13}C, 95−99%) into NAA by MA6244, nor was 2-^{13}C-indole itself incorporated into NAA. However, ring-^{14}C-anthranilic acid, a precursor of tryptophan, was readily incorporated by resting cells of MA6244 into NAA. In keeping with the established metabolism of anthranilic acid to indole-3-glycerol phosphate,[20] no incorporation of carboxyl ^{14}C-anthranilic acid into NAA was observed. The specific incorporation sites of anthranilic acid into NAA were established using ring-$^{13}C_6$-anthranilic acid and ^{15}N-anthranilic acid (Table 2). The aromatic carbons of anthranilic acid are indeed the precursor carbons for the aromatic carbons of the NAA indole nucleus, and the nitrogen of NAA is derived from the nitrogen atom of anthranilic acid (Figure 5).

NAA isolated from resting cells of MF6299 supplemented with [UL-$^{13}C_5$]-D-ribose resulted in a NMR spectrum resembling that obtained in the presence of 1,2-$^{13}C_2$-acetate, but at 5−10-fold less enrichment. This indirect labeling is in keeping with our earlier findings that ribose can act as a sole carbon source for the growth of MF6299. Thus, MF6299 must have the capacity to catabolize ribose through the pentose phosphate pathway to acetate, presumably via pyruvate and glyceraldehyde-3-phosphate. The acetate can then be cycled into mevalonic acid and into NAA biosynthesis. Incorporation of 1-^{13}C-D-ribose and [UL-$^{13}C_5$]-D-ribose into NAA clearly demonstrated that the remaining two carbons of NAA were not labeled by anthranilic acid, nor from acetate, but originate from C-1 and C-2 of ribose (Figure 5). This finding supports the hypothesis that the indole nucleus of NAA is formed from indole-3-glycerol phosphate in a fashion analogous to the biosynthesis of the indole core of tryptophan. It seems quite plausible then that indole-3-glycerol phosphate is a likely branch point between tryptophan and NAA biosynthesis.

The existence of a similar pathway was proposed in the biosynthesis of several plant phytoalexins, such as camalexin by *Arabidopsis thaliana* and the cyclic hydroxamic acid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) in maize.[21−23] In both of these cases, indole-3-glycerol phosphate appears to serve as the branch point between tryptophan and phytoalexin biosynthesis. Whether the indole-3-glycerol phosphate in NAA biosynthesis is first metabolized to indole which then reacts with GGPP, or if GGPP reacts directly with indole-3-glycerol phosphate, is unknown. Tryptophan synthases (TS) of plants and bacteria are quite similar. Both synthases exist as $\alpha_2\beta_2$ complexes wherein the α subunit which catalyzes the conversion

(19) Steyn, P. S.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1980**, 560−561.
(20) Kirschner, K.; Szadkowski, H.; Jardetzky, T.; Hager, V. *Methods Enzymol.* **1987**, *142*, 386−397.
(21) Tsuji, J.; Jackson, E.; Gage, D.; Hammerschmidt, R.; Somerville, S. C. *Plant Physiol.* **1992**, *98*, 1304−1309.
(22) Tsuji, J.; Zook, M.; Somerville, S. C.; Last, R. L.; Hammerschmidt, R. *Physiol. Mol. Plant Pathol.* **1993**, *43*, 221−229.
(23) Frey, M.; Chomet, P.; Glawisching, E.; Stettner, C.; Grun, S.; Winklmair, A.; Eisenreich, W.; Bacher, A.; Meeley, R. B.; Briggs, S.; Simcox, K.; Gierl, A. *Science* **1997**, *277*, 696−699.

of indole-3-glycerol phosphate to indole is a discrete subunit protein.[24,25] TSs of fungi, however, usually consist of a dimer of a single polypeptide that contains both the α and the β enzyme activities.[24,26] In procaryotes, indole generated by cleavage of indole-3-glycerol phosphate is confined to the tunnel that connects the α and β sites and is not released from tryptophan synthase.[27] However, in maize it was found that 2-^{14}C-indole and 3-^{13}C-indole were incorporated into DIMBOA, implying that free indole can gain access to the □ site of this fungal TS.[23,28] We found no incorporation of 2-^{13}C-indole directly into NAA, suggesting that either free indole does not have access to the β site of the nodulisporium TS or indole is not an intermediate in the biosynthesis of NAA.

Irrespective of which of these two mechanisms is functional in NAA biosynthesis, we propose that indole-3-glycerol serves as a branch point between tryptophan biosynthesis and NAA biosynthesis. This proposal eliminates the requirement for a tryptophan intermediate as suggested for penitrem A biosynthesis[14] and inferred for the other remaining members of this class of indole-diterpene natural products.[1,2,12,13]

Experimental Section

NMR Spectroscopy. All samples for NMR measurements were prepared by dissolving 15 μmol of sample in CD_3OD (99.6% CIL) and run under identical conditions. Spectra were acquired on a Varian Unity 400 at 100 MHz for ^{13}C and 400 MHz for ^1H, and were referenced to the solvent (CD_3OD) signal, 49.0 ppm (^{13}C) and 3.3 ppm (^1H). The ^{13}C and ^1H spectra of NAA were previously assigned.[1] ^{13}C spectra were acquired under the following conditions: 26 000 scans; 35° pulse width; 0.95 s acquisition time; 1 s relaxation delay; 7 kHz Waltz decoupling on the proton channel during acquisition and delay. FID data were processed by Fourier transform with 1 Hz exponential line broadening and baseline correction prior to integration. Integral areas were determined for selected regions up to 5*fwhm on either side of a peak maximum when separation from other peaks allowed. In the case of overlapping peaks, regions were extended to integrate the group of signals together. In most cases, enrichment factors were determined by comparison of the peak areas of labeled and unlabeled carbons in similar local environments. In many cases, incorporation levels were high enough (>3-fold over natural abundance) to be independently determined by comparison of the peak areas of an unlabeled carbon peak and its ^{13}C satellites arising from a neighboring spin label. The coupling constants measured from these satellites are shown in Table 1. In the double label experiment (1,2-$^{13}C_2$-acetate), intra- and interlabel couplings were present to such a degree that many previously resolved signals became intractable multiplets, preventing internal calibration of integral areas. In this case, the enrichment factors were determined by external calibration to a spectrum of NAA at natural abundance obtained under identical conditions.

Isotopes. Stable isotopes were obtained from the following: DL-mevalonolactone (2-^{13}C, 99%) from Isotec, Inc., acetic acid (2-^{13}C, 99%) sodium salt, L-tryptophan (indole-2-^{13}C, 95−99%), indole (2-^{13}C, 95−99%), anthranilic acid (ring-$^{13}C_6$, 99%), anthranilic acid (^{15}N, 98%), D-ribose (1-^{13}C, 99%) and D-ribose (UL-$^{13}C_5$, 98%) from Cambridge Isotope Laboratories, and acetic acid (1,2-$^{13}C_2$, 99%) sodium salt, from Sigma Chemical Co. Radioisotopes were obtained from the following: anthranilic acid (ring-UL-^{14}C, 12.5 mCi/mmol) from Sigma Chemical Co., anthranilic acid (carboxyl-^{14}C, 54 mCi/mmol) from American Radiolabeled Chemicals, Inc., and l-tryptophan (UL-^{14}C, 238 mCi/mmol) from Amersham.

Fermentation. Nodulisporic acid A producing cultures MF6244 and MF6299 were stored as frozen vegetative mycelia in seed medium containing 10% glycerol. Seed medium S-3 consisted of D-glucose, 50 g/L; monosodium glutamate, 10 g/L; amicase (Sheffield Products), 2.0 g/L; MES buffer, 20 g/L; NH_4Cl, 3.0 g/L; K_2HPO_4, 1.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $CaCO_3$, 1.0 g/L; and 20 mL of a trace mineral mixture (TE) composed of $ZnSO_4 \cdot 7H_2O$, 0.5 g/L; $FeSO_4 \cdot 7H_2O$, 0.5 g/L; $MnSO_4 \cdot H_2O$, 0.1 g/L; $CuSO_4 \cdot 5H_2O$, 0.5 g/L; and $CoCl_2 \cdot 6H_2O$, 0.04 g/L dissolved in 0.6 N HCl, and was adjusted to pH 6.0 before autoclaving. Growth was initiated by inoculating 50 mL of S-3 seed medium in a 250 mL nonbaffled flask with 1.0 mL of frozen vegetative cells and incubating the culture at 220 rpm and 29 °C for 48 h. One milliliter of seed culture was used to inoculate 40 mL of FFL-1 production medium consisting of D-glucose, 70 g/L; glycerol, 20 g/L; monosodium glutamate, 10 g/L; amicase, 8.0 g/L; lactic acid, 5.0 mL of 85% solution; MES buffer, 20 g/L; NH_4Cl, 3.0 g/L; K_2HPO_4, 1.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $CaCO_3$, 1.0 g/L; and 20 mL of TE. Ribose additions were made into FFL-1M production medium consisting of D-glucose, 80 g/L; glycerol, 100 g/L; monosodium glutamate, 20 g/L; amicase, 2.0 g/L; MES buffer, 20 g/L; NH_4Cl, 3.0 g/L; K_2HPO_4, 0.75 g/L; $MgSO_4 \cdot 7H_2O$, 1.0 g/L; $CaCO_3$, 1.0 g/L; and 20 mL of TE.

Washed Cell Incubations. After growth in production medium for approximately 5 days, or when NAA reached a titer of 20−30 μg/mL, the centrifuged mycelium was washed 2−3 times with 1.0 mM MOPS buffer, pH 6.5 and resuspended to the original volume in 20 mM MOPS buffer, pH 7.0 and 40 g/L glycerol. All labeled substrates were adjusted to pH 6.5−7.0 before addition to resting cells. 2-^{13}C-Acetate was added to washed cells of MF6244 in four additions of 10 mM each at 0, 21, 45, and 69 h, and the cells were harvested at 98 h. Similarly, 10 mM 2-^{13}C-mevalonolactone was added in four 2.5 mM aliquots. Ring-$^{13}C_6$-anthranilic acid and ^{15}N-anthranilic acid were added to separate preparations of MF6299 washed cells in three additions of 0.83 and 1.0 mM, respectively, at 0, 20, and 42 h. Cells were harvested at 72 h. 1-^{13}C-D-Ribose and [UL-$^{13}C_5$]-D-ribose were added in four additions of 1.0 and 1.4 mM, respectively, at 0, 24, 48, and 72 h to separate preparations of MF6299 cells grown in a FFL-1M medium. The cells were harvested at 96 h.

Isolation of NAA. NAA was extracted in crude form by the addition of 1.2 volumes of MEK per volume of washed cell incubation. The mixture was vigorously shaken for 45 min and centrifuged. The upper organic layer was removed and evaporated under vacuum. The aqueous residue was extracted with two volumes of ethyl acetate, and the organic layer was dried. The residue was dissolved in 80% MeOH, filtered, and purified by preparative HPLC using a Waters Symmetry Prep 19 × 300 mm column equilibrated at 40 °C with a flow rate of 8.0 mL/min. NAA was eluted with a MeOH/H_2O gradient consisting of 80% MeOH for 3 min followed by a linear gradient to 90% MeOH over 37 min. Preparative HPLC detection was at 290 nm. The fractions containing NAA were pooled, MeOH was removed under vacuum, and NAA was extracted from the residual H_2O with two volumes of ethyl acetate. Purified NAA was obtained by removal of ethyl acetate and drying under vacuum. The purity of each sample was assessed by dividing the calculated NAA concentration, as determined by HPLC analysis utilizing an external NAA sample of 96.5% purity, by the weight of the dried product.

Supporting Information Available: ^{13}C NMRs of all labeled NAAs, and the structure of hinnuliquinone (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017183P

(24) Miles, E. W. In *Advances in Enzymology and Related Areas of Molecular Biology*; Meister, A., Ed.; John Wiley & Sons: New York, 1991; Vol. 64, pp 93−173.
(25) Radwanski, E. R.; Zhao, J.; Last, R. L. *Mol. Gen. Genet.* **1995**, *248*, 657−667.
(26) Burns, D. *J. Biol. Chem.* **1989**, *264*, 3840−3848.
(27) Mathews, C. K.; van Holde, K. E. *Biochemistry*; The Benjamin/Cummings Publishing Co., Inc.: Redwood City, CA, 1990; p 719.
(28) Desai, S. R.; Kumar, P.; Chilton, W. S. *Chem. Commun.* **1996**, 1321.