3-Amino-4-hydroxybenzoic Acid Is Derived from the Tricarboxylic Acid Cycle Rather Than the Shikimic Acid Pathway[1]

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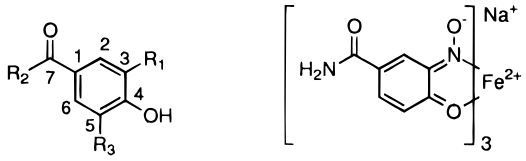
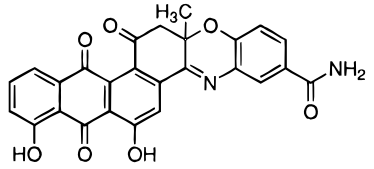
Abstract: The biosynthesis of 4-hydroxy-3-nitrosobenzamide in *Streptomyces murayamaensis* mutants MC2 and MC3 has been studied using sodium [1,2-$^{13}C_2$]- and [1-^{13}C,$^{18}O_2$]acetate, sodium [2,3-$^{13}C_2$]succinate, [1,2-$^{13}C_2$]glutamic acid, [4-^{13}C]aspartic acid, and sodium [1-^{13}C]- and [2,3-$^{13}C_2$]pyruvate. ^{13}C NMR analysis of the labeling patterns from the first two of these suggested a pathway via condensation of a four-carbon unit from the tricarboxylic acid (TCA) cycle with a three-carbon unit, possibly phosphoenol pyruvate. Subsequent specific incorporations of the labeled succinic acid, aspartic acid, and glutamic acid confirmed the TCA cycle involvement and the orientation of the four-carbon intermediate. Specific incorporation of the labeled pyruvic acids confirmed the involvement of a three-carbon unit and defined its orientation. This is the first aminohydroxybenzoic acid derivative shown not to be derived from a shikimic acid-type pathway, and its origin provides a rationale for the biosynthesis of other microbial products such as asukamycin, manumycin, and the michigazones.

We have recently described the isolation of 4-hydroxy-3-nitrosobenzamide, **1**, and its ferrous chelate, **2**, from *Streptomyces murayamaensis*,[2] as well as a metabolite, murayaanthraquinone, **3**, apparently derived from condensation of a 3-amino-4-hydroxybenzamide (**4**) equivalent and a benz[*a*]anthraquinone.[3] The free acid,[4] *p*-hydroxystyryl ester,[5−7] and aldehyde[8,9] analogs of **2** had been previously reported. We have also reported that 3-amino-4-hydroxy[5-^{2}H]benzoic acid, **5a**, was readily incorporated into **2** by *S. murayamaensis*.[2] We now report on the biosynthesis of **2** and, therefore, of **5** via the tricarboxylic acid (TCA) cycle and a three-carbon unit from glycolysis.



1, R_1 = NO, R_2 = NH_2, R_3 = H
4, R_1 = NH_2, R_2 = NH_2, R_3 = H
5, R_1 = NH_2, R_2 = OH, R_3 = H
5a, R_1 = NH_2, R_2 = OH, R_3 = D

2



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Results and Discussion

During the course of an exploratory feeding of sodium [1-^{14}C]-acetate to *S. murayamaensis* mutant MC3 in order to study the biosynthesis of murayaquinone,[10] a polyketide metabolite which is overproduced by this mutant strain,[11] it was found that both murayaquinone and **2** were significantly radioactive. A sample of sodium [1,2-$^{13}C_2$]acetate, **6a**, was fed next and the labeled **2a** analyzed by 1D ^{13}C NMR spectroscopy[12] and by a 2D INADEQUATE experiment. The former spectrum revealed pairs of large satellites (∼2% enrichments) flanking resonances for C-1, C-5, C-6, and C-7, and much less intense satellites (∼0.5% enrichments) flanking the C-2, C-3, and C-4 resonances. The latter spectrum contained cross-peaks between C-1 and C-7 and between C-5 and C-6, revealing that each pair represented incorporation of a two-carbon acetate unit. While cross-peaks were not observable for any of the other three resonances, coupling between C-3 and C-4 was confirmed with a ^{13}C{^{13}C} (COSYX) experiment. The coupling between C-2 and C-3 could not be detected directly in this sample. The combined data suggested the possible presence of biogenetic units of four carbons and three carbons, each coming from a significantly different *in vivo* pool.

Sodium [1-^{13}C,$^{18}O_2$]acetate, **6b**, was fed next and yielded **2b**. ^{13}C NMR analysis showed that, in addition to enrichment of C-7 (δ 167.5, the carboxyl carbon), C-5 (δ 120.8) rather than C-6 (δ 135.7) was enriched. A second resonance was also

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(1) Portions of this work were presented at Pacifichem '95, Honolulu, Hawaii, December 18, 1995.

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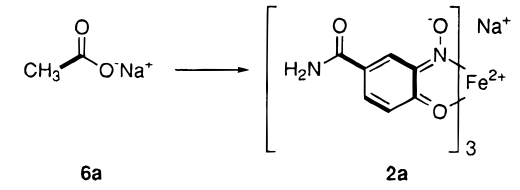
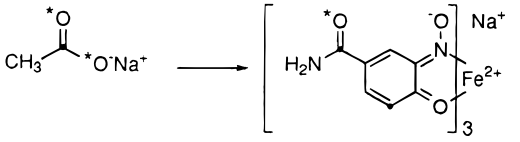


6a **2a**

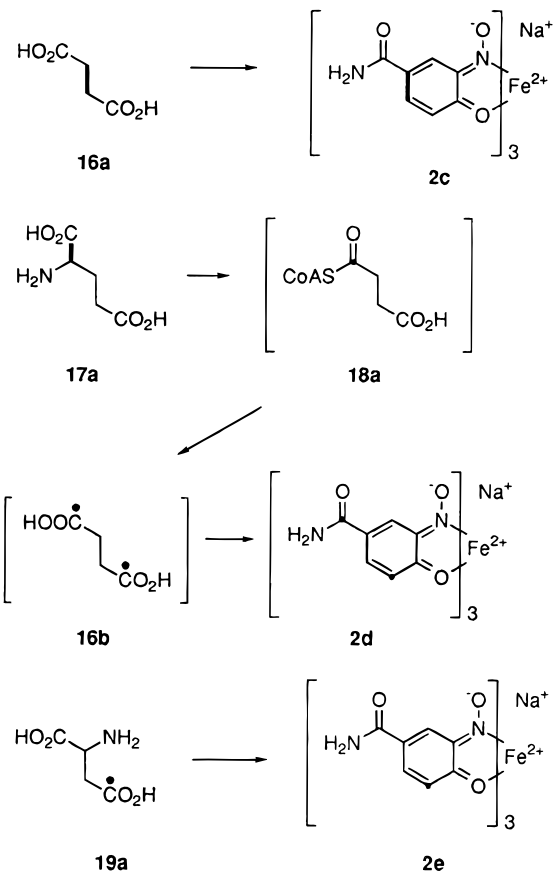


6b **2b**

observed for C-7 (3.2 Hz upfield isotope shift), indicating the presence of one ^{18}O label at this site. An average enrichment of 10% was measured for C-7 and C-5, and from the height of the ^{18}O-shifted resonance, a 16% retention of ^{18}O could be estimated. No clear enrichment of the other three carbons could be discerned. Most importantly, from the ^{13}C enrichments it was clear that the acetate-derived units at C-1/C-7 and C-5/C-6 were linked tail-to-tail, rather than head-to-tail. Such tail-to-tail coupling is the signature of the TCA cycle.

Previous biosynthetic studies have shown that 2-amino-3-hydroxybenzoic acid, **7**,[13] 2-amino-6-hydroxybenzoic acid, **8**,[14] and 3-amino-5-hydroxybenzoic acid, **9**,[15,16] are derived from variations of the shikimic acid pathway *via* either dehydroshikimic acid (**10**, R = OH) or aminodehydroshikimic acid (**11**, R = NH_2) (Scheme 1; the heavy bonds shown are those that remain intact from glucose, **12**). This pathway involves the condensation of a three-carbon unit, phosphenolpyruvate (PEP), **13**, with a four-carbon unit. For **10**, this is erythrose-4-phosphate, **14**, and for **11** it is apparently the imino analog **15**.[17]

The labeling observed for C-7/C-1/C-6/C-5 of **2** from the acetate feedings revealed the involvement of a four-carbon unit. In this case, however, it arises from a dicarboxylic acid of the TCA cycle rather than from erythrose-4-phosphate. The direct major labelings observed from the numerous precursors subsequently fed revealed the important features of the pathway to this new isomer of aminohydroxybenzoic acid (3,4-AHBA). These feedings were done using *S. murayamaensis* mutant MC2[18] to avoid the overproduction of murayaquinone. The involvement of the TCA cycle was confirmed directly by feeding sodium [2,3-$^{13}C_2$]succinate, **16a**, which yielded **2c** with ^{13}C coupling between C-1 and C-6, and by feeding [1,2-$^{13}C_2$]-glutamic acid, **17a**, which yielded **2d** with enrichments at C-5 and C-7. The succinate labeling of C-1 and C-6 (~1.5% enrichment) confirmed incorporation of an intact four-carbon unit. The glutamic acid had been converted *in vivo* to [1,2-$^{13}C_2$]-α-ketoglutarate and then to [1-^{13}C]succinyl CoA, **18a**, which was cleaved to the symmetrical succinic acid **16b** in the normal course of the TCA cycle. [4-^{13}C]Aspartic acid, **19a**, was fed next and was incorporated unsymmetrically to yield **2e**. Thus, the incorporation of **19a** defined the orientation of the four-carbon unit.



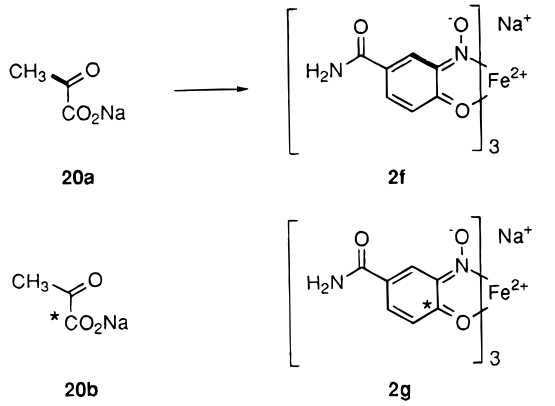
16a **2c**

17a **18a**

16b **2d**

19a **2e**

The involvement of a three-carbon unit was confirmed and its orientation defined by feeding sodium [2,3-$^{13}C_2$]- and [1-^{13}C]-pyruvates, **20a** and **20b**, respectively. The former yielded **2f**



20a **2f**

20b **2g**

with couplings between C-2 and C-3, while the latter yielded **2g** with C-4 enriched. Although a correlation for the C-2/C-3 coupling could not be observed in the COSYX or 2D INADEQUATE spectra of **2a**, this coupling was obvious in the spectrum of **2f** and was confirmed by a 2D INADEQUATE experiment.

As had been the case for incorporation of **6a**, additional enrichments were observed from many of the feeding experiments. These revealed details about pool sizes and the activity level of primary metabolic pathways in these mutant strains of *S. murayamaensis*. The various feedings with acetate and pyruvate demonstrated the presence of a relatively small steady-state pool of acetyl CoA and a very active flux into and through the TCA cycle (Scheme 2). Indeed, the first feeding with sodium [2,3-$^{13}C_2$]pyruvate actually gave higher enrichments of the four-carbon unit (*via* **16c** → **21a,b** → **2h,i**) than the three-

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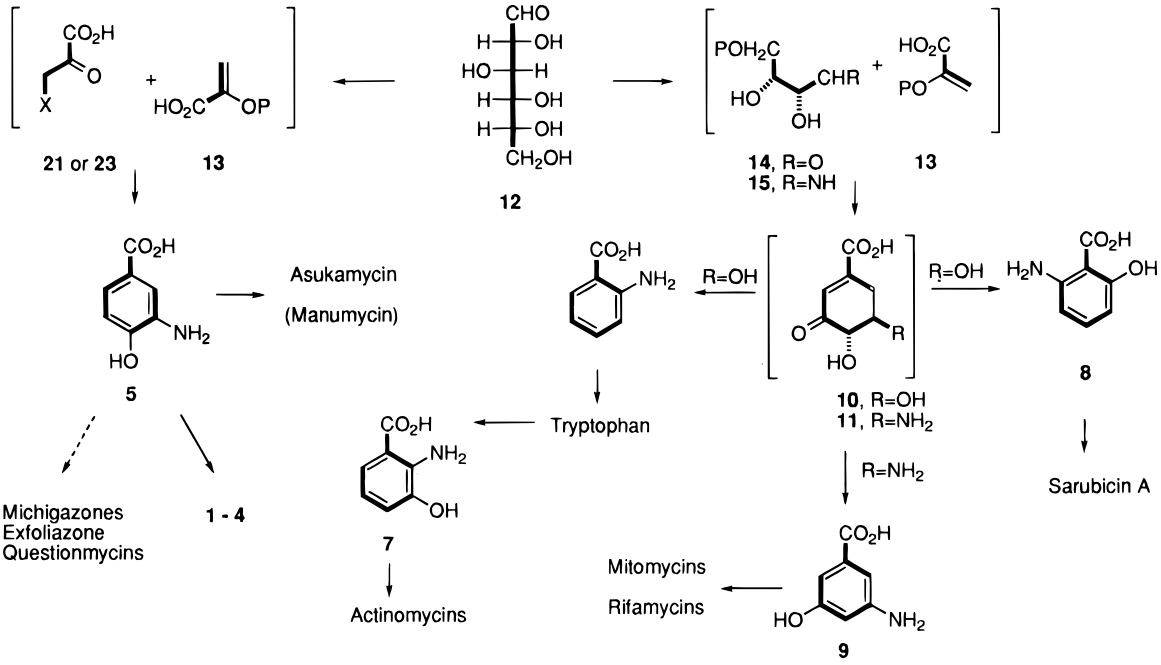
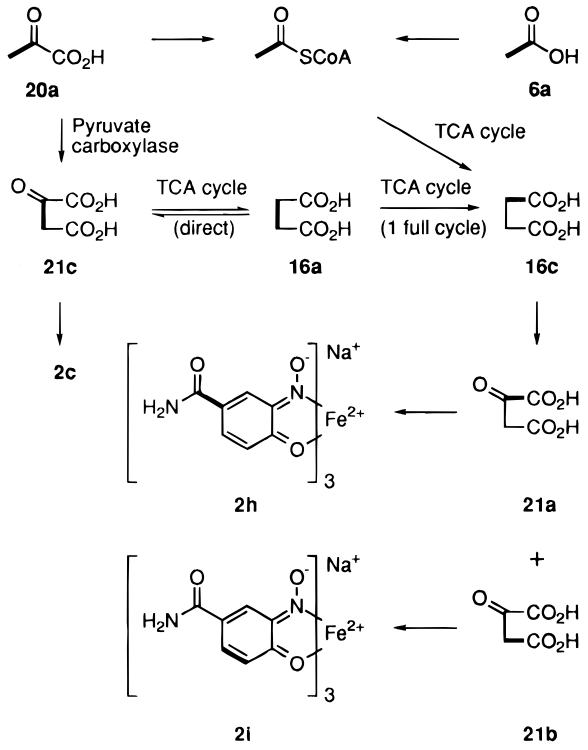
Scheme 1



Scheme 2



carbon unit. However, this trend was reversed when excess unlabeled acetate was cofed. When sodium [2,3-$^{13}C_2$]succinate was fed, moderate additional enrichments (evidenced by the coupled resonances) were observed at C-1/C-7 and C-5/C-6 (~0.5% enrichment, representing ca. one-third of the enrichments *observed* for C-1 and C-6). These additional labelings (e.g. **2h** and **2i**) can be explained by a full turn through the TCA cycle to yield **16c**.[19] [2,3-$^{13}C_2$]Oxaloacetate, **21c**, would have been generated from both **16a** and **20a**, leading to **2c**. In the former case, this was the major labeling, but in the latter case it would have been minor and masked by the large satellites at C-1 and C-6 from **2h** and **2i**, respectively. A low level of enrichment at C-7 was observed from feeding [4-^{13}C]aspartate, and is consistent with this scheme, as well; at least some was converted to [4-^{13}C]oxaloacetate and the label from some of this was symmetrized via succinate. The various incorporations are summarized in Table 1.

The activities of various metabolic routes were also revealed from the labelings of the three-carbon unit. In addition to the substantial incorporations into the four-carbon unit of **2**, [2,3-$^{13}C_2$]succinate, **16a**, gave a low level of labeling at C-2 and C-3 (~0.1%), which can be readily explained by conversion of **16a** to [2,3-$^{13}C_2$]oxaloacetate, **21c**, and its conversion to [2,3-$^{13}C_2$]PEP, **13a**, by PEP carboxykinase, leading to **2f** (Scheme 3). Sodium [2,3-$^{13}C_2$]pyruvate not only gave coupled intense satellites for C-2 and C-3 (**2f**) but also yielded very small satellites for C-4. The coupling constant indicated C-4, too, was coupled to C-3. This result can be explained by conversion of this pyruvate *via* the TCA cycle to oxaloacetate **21a** and then on to [1,2-$^{13}C_2$]PEP and **2j**. [3-^{13}C]PEP would also be formed and lead to [2-^{13}C]**2** (not shown). Finally, the spectrum of **2** derived from incorporation of [1-^{13}C]pyruvate also showed very small satellites for C-4 and C-5, which would be due to (1) the action of pyruvate carboxylase, (2) malate dehydrogenase and fumarase of the TCA cycle, which would presumably "symmetrize" the labeling of the four-carbon diacids, and (3) sufficiently small metabolite pools to afford observable statistical coupling between the the four-carbon and three-carbon intermediates.

The biosynthesis of **5,** the precursor to **2,** can be rationalized via a number of alternative pathways, converging on a common intermediate (**22**). As shown in Scheme 4 (heavy bonds identify the advanced biogenetic units), **13** could condense with oxaloacetate, **21**, or with an α-keto acid, **23**, derived from homoserine or methionine, both of which are derived from **21** *via* **19**. This condensation would yield **24**, which could undergo elimination of an appropriate leaving group, generating **22**. The same intermediate could be generated from **13** and vinyl glyoxylate, **25**.[20,21] Condensation with pyridoxamine phosphate could then provide the amino nitrogen, affording **26**. Cyclization, tau-

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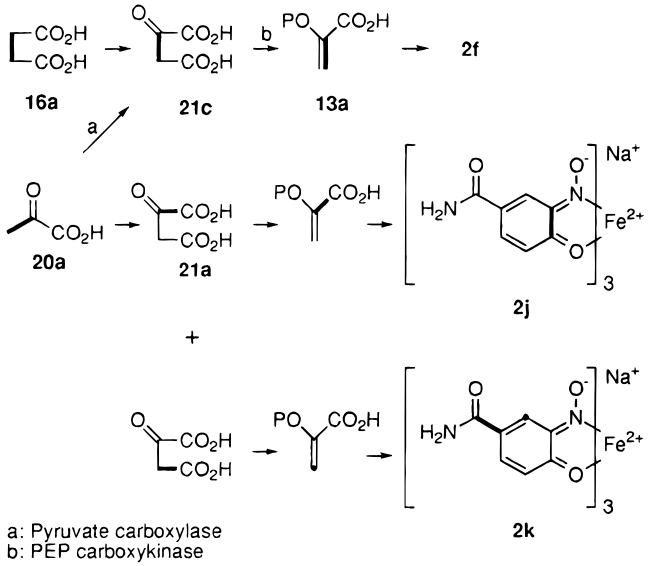
Table 1. ^{13}C NMR Results from the Incorporation of ^{13}C-Labeled Precursors into the Ferrous Chelate of 4-Hydroxy-3-nitrosobenzamide, **2**

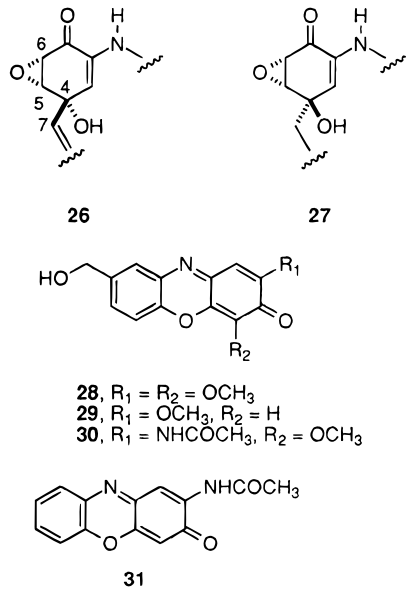
		6a		**6b**		**16a**				**20a**		**20b**	
carbon	δ	J_{CC} (Hz)	% enrich.	$\Delta\delta$ (Hz)	% enrich.	J_{CC} (Hz)	% enrich.	**17a**: % enrich.	**19a**: % enrich.	J_{CC} (Hz)	% enrich.	J_{CC} (Hz)	% enrich.
1	119.5	64.2	1.6			56.9	1.2			64.3	0.8		
2	110.2	63.4	0.4			62.2	0.1			63.5	0.7		
3	158.7	64.2	0.6			64.1	0.1			63.2	0.6		
4	179.8	64.1	0.7							63.8	0.1	62.2	0.9
5	120.8	61.0	1.3		10.0	59.2	0.5	2.2	0.6	59.7	0.6	61.5	0.2
6	135.7	61.2	1.4			57.1	1.8			58.4	0.8		
7	167.5	64.3	1.2	3.2	10.0	64.4	0.5	0.6	0.5	64.4	0.7	64.3	0.3

Scheme 3



tomerization, and hydrolysis would ultimately yield **5**. We have previously shown that 2-amino-6-hydroxybenzoic acid, **8**, is first converted to its carboxamide before incorporation into sarubicin A,[22] and this may be the next step in the conversion of **5** to **1** and **2**.

While it will be necessary to establish the precise metabolites from aspartic acid and pyruvic acid for the four- and three-carbon intermediates in the biosynthesis of **5**, it is clear that the pathway is not another offshoot of the shikimic acid pathway. Scheme 1 shows how this fits into an overall perspective of AHBA biosyntheses from glucose. With the biosynthetic parameters so far established, it appeared that **5** may be involved in more than just those pathways revealed in *S. murayamaensis*. Thus, Floss *et al.* had observed the same carbon labeling patterns of the *m*-C_7N moieties, **26** and **27**, of asukamycin and manumycin, respectively, from feeding **6a** and from feeding sodium [1-^{13}C]acetate, as well as a consistent labeling pattern from feeding sodium [1,2-$^{13}C_2$]succinate, although they were unable to detect any labeling of the three-carbon unit from these feedings.[23] It seemed reasonable to us that this may simply have been a matter of internal metabolite pool sizes. Thiericke *et al.* tested potential aromatic intermediates, but neither [^{13}COOH]-3-aminobenzoic acid nor [^{13}COOH]-**9** labeled asukamycin, while each of these materials yielded new, labeled analogs of manumycin, apparently by directed biosynthesis.[23,24] It therefore appeared possible that 3-amino-4-hydroxybenzoic acid, **5**, would be an intermediate in the biosynthesis of manumycin and of asukamycin, with oxidative modification of the *m*-C_7N unit occurring after addition of the polyketide portions. Indeed, when a sample of **5a** was recently fed to *S. nodusus* ssp. *asukaensis*, it was incorporated into asukamycin.[25] It is reasonable to expect a similar result with manumycin. The phenoxazones michigazone,[26] **28**; 4-demethoxymichigazone,[27] **29**; exfoliazone,[28] **30**; and *N*-acetylquestiomycin,[29] **31**, should also derived from 3,4-AHBA.



Experimental Section

General. Media were prepared with deionized water. Sodium [1,2-$^{13}C_2$]- and [1-^{13}C,$^{12}O_2$]acetate, [2,3-$^{13}C_2$]succinic acid, [1,2-$^{13}C_2$]-glutamatic acid, [4-^{13}C]aspartic acid, and [1-^{13}C]- and [2,3-$^{13}C_2$]pyruvic acid were obtained from Cambridge Isotope Laboratories, Inc. Compounds were dissolved in H_2O (the pH was then adjusted to neutrality as needed) and introduced in equal aliquots through a 2 μm syringe filter.

HPLC Analysis of 3-Amino-4-hydroxybenzamide Ferrous Chelate (2). A Waters NovaPak C_{18} radial compression column (0.8 × 10 cm, 4 μm bead size) was eluted at a flow rate of 1.5 mL/min with Milli-Q 5% water/acetonitrile containing 0.1% acetic acid. Absorption spectra were obtained using a Waters 990+ diode array detector with 2 nm resolution over a range of 200−650 nm. The retention time for **2** was 5.1 min.

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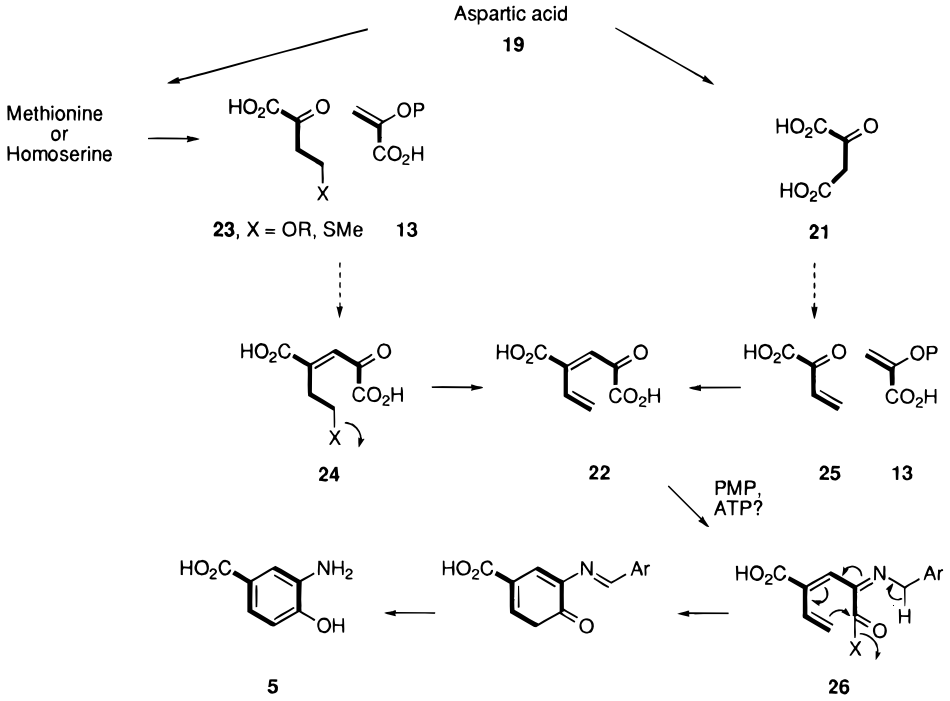
Scheme 4



Production of 2. A Kinako soybean−glycerol seed culture[30] (50 mL in a 500 mL foam stoppered Erlenmeyer flask) was inoculated with frozen agar plugs for strain MC2 and with agar plugs of actively growing mycelium for strain MC3, and each was incubated on a rotary shaker at 27 °C, 280 rpm for 48 h. Production media were composed of 200 mL of 4% farina plus trace metals[31] in 2 L flasks for strain MC2 and 400 mL of glycerol−ammonium sulfate medium[30] in 2 L foam-stoppered flasks for strain MC3. Each was inoculated at 5% v/v with seed culture.

Fermentation Protocol for Strain MC3. Precursors were fed in the following manner: sodium [1,2-$^{13}C_2$]acetate (**6a**, 102.2 mg) and sodium [1-^{14}C]acetate (3.00 μCi) dissolved in H_2O (3.00 mL) were introduced to a 400 mL culture of *Streptomyces murayamaensis* mutant MC3 in equal portions 14, 20, and 26 h after inoculation. Sodium [1-^{13}C,$^{18}O_2$]acetate (**6b**, 154.2 mg) and sodium [1-^{14}C]acetate (3.00 μCi) dissolved in H_2O (50.0 mL) were added to two 400 mL culture broths in equal portions 14, 17, 20, 23, and 27 h after inoculation. After a total of 36 h, the whole broth was diluted with an equal volume of EtOAc and the mixture sonicated. After filtration, the resulting aqueous layer was adjusted to pH 2−3 and re-extracted with EtOAc (2 × 200 mL). Extraction of the remaining aqueous layer with *n*-BuOH (2 × 200 mL) and concentration yielded a solid residue (42 mg). This was dissolved in 5% H_2O/MeOH and was chromatographed on a 2.5 × 15 cm Sephadex LH-20 column, prepared, and eluted with the same solvent, to give 8.1 mg of **2a**. An estimated production of 12.6 mg was calculated by analysis of the area of peak corresponding to **2a** in the HPLC trace of the initial extract monitored at 254 nm. Workup of the **6b** feeding yielded 54.2 mg from the butanol extraction. Sephadex LH-20 purification of this material gave 16.7 mg of **2b**. An estimated production of 27.1 mg was calculated by HPLC analysis.

Fermentation Protocol for Strain MC2. In separate experiments, [2,3-$^{13}C_2$]succinic acid, **16a,** (102 mg); [2,3-$^{13}C_2$]-L-glutamic acid, **17a** (37.5 mg); sodium [2.3-^{13}C]pyruvate, **20a** (84 mg); sodium [2,3-$^{13}C_2$]-pyruvate (120 mg) plus sodium acetate (500 mg); and sodium [1-^{13}C]-pyruvate, **20b** (120 mg) plus sodium acetate (500 mg) were fed to production cultures (5 × 200 mL) in four equal portions at approximately 12, 16, 20, and 24 h after inoculation. [4-^{13}C]-DL-Aspartic acid, **19a** (245 mg), was fed in one portion 12 h after inoculation. After a total of 60 h, the cultures were centrifuged, and the broth was adjusted to pH 3 with 1 N HCl and then extracted with EtOAc.[30] The organic extracts were washed with saturated brine and partitioned against aqueous ferrous sulfate, and the bright green aqueous layer was washed with EtOAc. NaCl was then added to near saturation and the mixture extracted with *n*-BuOH. The butanol extracts were washed with saturated brine and concentrated *in vacuo*. The green solid was dissolved in MeOH (10−20 mL), filtered, diluted with water (5% v/v), and chromatographed on Sephadex LH-20 as described above. The yields were as follows: **2c**, 33.5 mg; **2d**, 61.2 mg; **2e**, 13.2 mg; **2f**, 34.0 mg; **2g**, 45.0 mg.

^{13}C−^{13}C Coupling Experiments for 2a. INADEQUATE spectra (Bruker AM 400, DMSO-d_6) were obtained using the following acquisition parameters (Bruker pulse program INAD2D.AUR): D1 2.0; P9 90; S1 15H; D3 0.002; S2 15H; P1 8.3; D2 0.00550; P2 16.6; RD 0.0; PW 0.0; D0 3 ms; NE 128; ND0 2; MC2 M; SI2 2048 W; SI1 512 W; NS 128 × 2; WDW2,1 Q; SSB2,1 2. COSYX spectra were obtained using the following acquisition parameters (COSYX.AUR): D1 1.0; P9 90; S1 15H; D3 0.002; S2 15H; P1 8.3; D2 0.00550; P2 8.3; RD 0.0; PW 0.0; D0 3 ms; NE 128; ND0 1; MC2 M; SI2 2048 W; SI1 1024 W; NS 736; WDW2,1 S; SSB2,1 0.

^{13}C−^{13}C Coupling Experiment for 11a. Parameters for INAD2D. AUR (Bruker AC-300) were as follows: D1 2.5; P9 100; S1 15H; D3 0.003; S2 15H; P1 7.0; D2 0.005; P2 14.00; RD 0.0; PW 0.0; DO 3 ms; NE 128; NDO 1; MC2 M; SI2 2048 W; SI1 256 W; NS 128 × 2; WDW2,1 Q; SSB2,1 2.

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