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# BIOSYNTHESIS OF BROMINATED TYROSINE METABOLITES BY *APLYSINA FISTULARIS*[1]

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ABSTRACT.—The biosynthesis of brominated tyrosine metabolites by the marine sponge *Aplysina fistularis* was investigated. [*U*-$^{14}$C]-L-Tyrosine, [*U*-$^{14}$C]-L-3-bromotyrosine, and [*U*-$^{14}$C]-L-3,5-dibromotyrosine were incorporated into both dibromoverongiaquinol [**1**] and aeroplysinin-1 [**2**], and [*methyl*-$^{14}$C]methionine was specifically incorporated into the *O*-methyl group of **2**. [*Methyl*-$^{14}$C]-L-*O*-methyltyrosine, [*methyl*-$^{14}$C]-L-3,5-dibromo-*O*-methyltyrosine, and several putative nitrile precursors were not incorporated into **1** or **2**.

Dibromoverongiaquinol [**1**] (1,2) and aeroplysinin-1 [**2**] (3–6) were two of the first examples of compounds biogenetically derivable from halogenated tyrosine isolated from sponges of the order Verongida. Subsequently, over 100 halotyrosine-derived metabolites have been reported from sponges of this order.

Early biosynthetic studies of **1**, **2**, and aerothionin (for a partial structure, see Scheme 7) by DeRosa, Minale, and Sodano were unsuccessful (7). *Aplysina aerophoba* was fed $^{14}$C-labeled Tyr, Orn, and Met, using a one- or two-day incubation period, but no incorporation of these precursors into **1**, **2**, or aerothionin was observed.

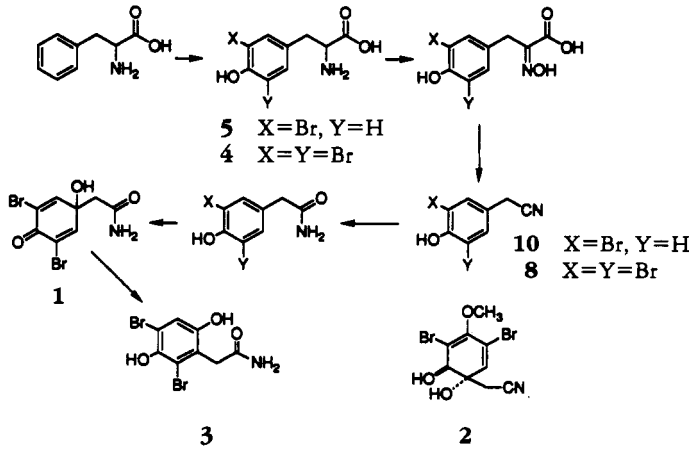
Our group later investigated the biosynthesis of dibromotyrosine-derived metabolites in *A. fistularis* and demonstrated the conversion of Phe and Tyr to **1** as well as to the rearranged product, dibromohomogentisamide [**3**] (8). In addition to implying that the sponge can convert Phe into Tyr, the comparable radioactivity found in **1** and **3** supports the hypothesis that **3** is formed from **1** via a skeletal rearrangement analogous to the mammalian catabolism of Tyr to homogentisic acid. Double labeling studies also revealed that the sponge can convert the side-chain in Phe to the acetamide side-chain in **3** without deamination. In addition to corroborating the known occurrence of bromophenol nitriles and oximes in Verongid sponges, this work supported the biosynthetic pathway in Scheme 1, with **2** being formed by a related pathway (see below) (8). Moreover, it has been demonstrated that α-oximino acids undergo facile dehydration/decarboxylation to give nitriles in vitro (9) and in vivo (10), that 4-hydroxy-2,5-cyclohexadienone-4-acetic acid is converted to homogentisic acid (11), and that **1** is converted to **3** (12) in aqueous alkali, further supporting the proposed conversion.

## RESULTS AND DISCUSSION

In the present investigation the biosynthesis of brominated tyrosine metabolites in *A. fistularis* has been further studied by preparing and conducting feeding experiments with more advanced labeled precursors. A number of feeding experiments involving a variety of labeled brominated and *O*-methylated derivatives of tyrosine and 4-hydroxybenzyl cyanide have been completed.

Because we and others (13) encountered difficulties reproducing the literature preparation of 3,5-$Br_2$Tyr [**4**] (14), a procedure was developed in which **4** can be prepared in yields of greater than 90% (15). A suspension of Tyr in HOAc was heated at 60° and stirred overnight in the presence of a slight excess of $Br_2$. The synthesis was readily scaled down to give radiochemically pure **4** in 100% radiochemical yield with the desired specific activity. The most efficient synthesis of 3-BrTyr [**5**] was bromination of Tyr with

[1]This article is dedicated to Professor Paul Scheuer in the year of his 80th birthday.



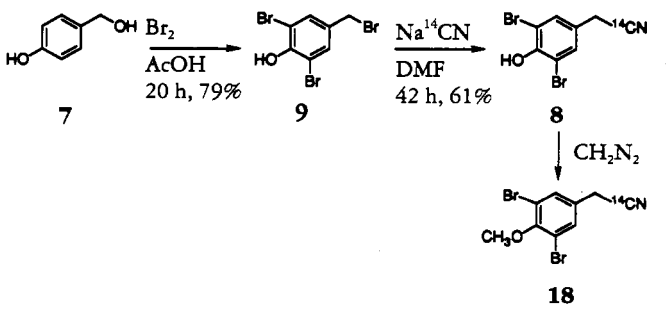

SCHEME 1

aqueous $KBrO_3$ and KBr (16). Trace amounts of Tyr and $Br_2$Tyr were easily separated from the desired product with a Sephadex G-15 column to give a 96% yield of **5**. Radiochemically pure labeled **5** was obtained in 92% radiochemical yield by scaling down the same procedure.

Nucleophilic substitution with $^{14}C$-labeled cyanide on an appropriate benzyl substrate would be a suitable general approach to the desired labeled nitriles. Schwartz reported a convenient synthesis of 4-hydroxybenzyl cyanide [**6**] using 4-hydroxybenzyl alcohol [**7**] as the substrate (17) and we planned a similar approach to 3,5-dibromo-4-hydroxybenzyl cyanide [**8**], via 3,5-dibromo-4-hydroxybenzyl alcohol. However, bromination of **7** in HOAc gave a 79% yield of 3,5-dibromo-4-hydroxybenzyl bromide [**9**] instead of the expected alcohol. Reaction of **9** with a slight excess of NaCN in DMF at room temperature then gave **8** in 72% yield.

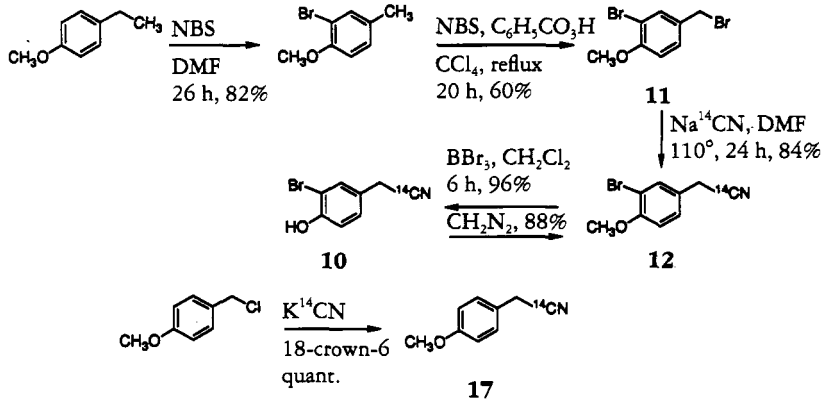
Radiochemically pure $^{14}C$-labeled **8** was similarly prepared in 61% radiochemical yield after prep. hplc. Treatment of $^{14}C$-labeled **8** with excess $CH_2N_2$ gave 178 μCi of radiochemically pure **18** (100% radiochemical yield) (Scheme 2).

Synthesis of $^{14}C$-labeled 3-bromo-4-hydroxybenzyl cyanide [**10**] required a hydroxybenzyl halide substrate with a phenol protecting group that could be removed under conditions which would not hydrolyze or reduce the nitrile. The bromide [**11**] was synthesized by aromatic bromination of *p*-methylanisole with *N*-bromosuccinimide (NBS) in DMF followed by benzylic bromination with NBS and catalytic benzoyl peroxide in $CCl_4$. Heating a mixture of NaCN and **11** in DMF at 110° overnight provided unlabeled 3-bromo-4-methoxybenzyl cyanide [**12**] in 82% yield (15). $^{14}C$-




SCHEME 2

Labeled **12** was similarly prepared with a radiochemical yield of 84% after prep. hplc. Demethylation of **12** was effected efficiently with $BBr_3$ in $CH_2Cl_2$ to give a 96% radiochemical yield of labeled **10** after prep. hplc. The overall radiochemical yield of **10** based on $^{14}C$-labeled cyanide was 80% (Scheme 3). A sample of $^{14}C$-labeled 3-bromo-4-hydroxybenzyl [$^{14}C$]cyanide [**10**] could also be converted with excess $CH_2N_2$ back to the desired labeled *O*-methyl nitrile **12**. Prep. hplc was required to remove small amounts of radiochemical impurities to give 43 μCi of radiochemically pure labeled **12** (88% radiochemical yield).

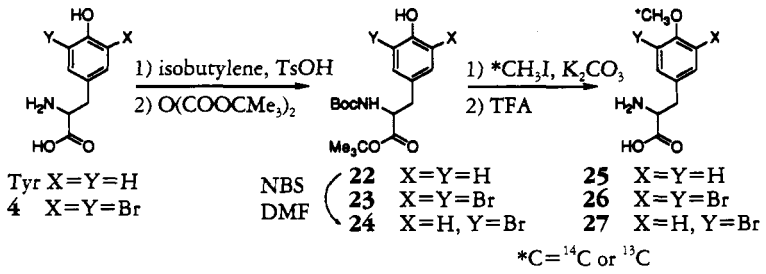



SCHEME 3

A 102% radiochemical yield of radiochemically pure 4-methoxybenzyl-[$^{14}C$]cyanide [**17**] was obtained by stirring at room temperature a mixture of *p*-methoxybenzyl chloride, $K^{14}CN$, and an equivalent of 18-crown-6 in MeCN.

The approach to synthesizing *O*-methyl tyrosines $^{14}C$-labeled in the *O*-methyl group was to use $^{14}C$-methyl iodide with a suitably protected Tyr substrate. The *t*-butyl esters of Tyr and $Br_2$Tyr were prepared in yields of 58% and 48%, respectively, by treating the amino acids with isobutylene and *p*-toluenesulfonic acid in dioxane (18). The Boc group was added in yields of 96% [**22**] and 82% [**23**], respectively, with di-*t*-butyl dicarbonate. Monobromo Boc-Tyr *t*-butyl ester was prepared by bromination of **22** with NBS in DMF to give **24** in 62% yield (Scheme 4).

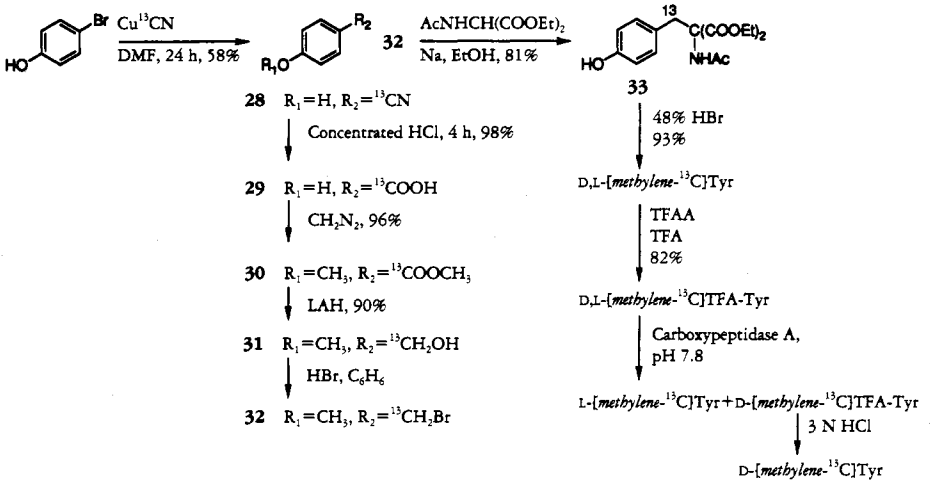
The $^{14}C$-labeled *O*-methyltyrosines **25** and **26** were prepared from the substrates **22** and **23** in radiochemical yields of 74% and 84%, respectively, by treating them with $K_2CO_3$, $^{14}CH_3I$, and 18-crown-6 in $C_6H_6$, followed by trifluoroacetic acid. $^{13}C$-Labeled **25–27** were prepared in a similar fashion in the hope that incorporations of these




SCHEME 4

precursors into **1** and **2** would be great enough for nmr or ms detection (which was not the case).

We also hoped that under optimum culture conditions incorporation of Tyr into **1** and **2** would be great enough for stable isotope experiments. [*Methylene*-$^{13}$C] tyrosine was prepared as outlined in Scheme 5. Cuprous [$^{13}$C]cyanide was prepared as described by Meinert (19) and the $^{13}$C-labeled nitrile **28** was synthesized in 58% yield from 4-bromophenol and cuprous [$^{13}$C]cyanide in refluxing DMF. Nitrile **28** was hydrolyzed with concentrated HCl to give [*carboxy*-$^{13}$C]-*p*-hydroxybenzoic acid (**29**, 98%), which was treated with excess $CH_2N_2$ to give methyl [*carboxy*-$^{13}$C]-*p*-methoxybenzoate [**30**] in 96% yield. The ester was reduced with $LiAlH_4$ in $Et_2O$ to give [*methylene*-$^{13}$C]-*p*-methoxybenzyl alcohol [**31**] in 90% yield, **31** was converted to benzyl bromide **32** by treatment with excess hydrogen bromide in $C_6H_6$, and **32** reacted with sodium and diethyl acetamidomalonate in EtOH to give **33** in 81% yield. The racemic diester **33** was then hydrolyzed in 93% yield to give racemic [*methylene*-$^{13}$C]tyrosine, which was converted to its *N*-trifluoroacetyl derivative (82%). The latter compound was resolved by treating with carboxypeptidase A to give L-[*methylene*-$^{13}$C]tyrosine in 88% yield (20) plus *N*-TFA-D-Tyr, which was recovered from the enzymatic reaction and hydrolyzed with 3 N HCl to give a 60% yield of D-[*methylene*-$^{13}$C]tyrosine. Both labeled tyrosine enantiomers were determined to be greater than 99% optically pure by chiral GC analysis.




SCHEME 5

*Aplysina fistularis* is a bright yellow to orange sponge which turns rapidly purple and eventually black when it is dying (21). It has a high respiration rate (22), and even in highly aerated $H_2O$, the sponges could not be kept in a one-gallon closed system for more than three days without showing stress or dying. However, they could be maintained for at least a month in one-gallon glass jars kept in the dark and receiving a continuous flow of sea water aerated with a standard aquarium pump. Our approach then was to add the precursors as a solution to the closed system within 2 or 3 days, then either (a) terminate the experiment after a short period, or (b) add a continuous flow of fresh sea water to the aquarium for a longer period (1 or 3 weeks).

Sponge samples were collected by chipping away the rock substrate that the organisms were attached to, then handled as little as possible. Specimens were transferred

immediately to the Catalina Marine Science Center without exposure to air. Sponges, 30–75 g squeezed dry wt, were placed in one-gallon jars containing three liters of aerated, unfiltered sea water for each feeding experiment. An antibiotic mixture was added to each jar, and after two to three hours, a continuous flow of fresh running sea water was passed through each jar. The specimens were left overnight, and if they appeared healthy the following day, a solution of the precursor was added in one portion to the closed, highly aerated system. The experiment was either terminated after three days, or after two days the system was opened to fresh running sea water for one or three weeks, and then terminated. Sponges looked quite healthy at the end of all feeding experiments, with some of the sponges in the three-week experiments attaching themselves to the glass jars.

The brominated tyrosine sponge metabolites were isolated (with **1** and/or **2** as the major metabolites, which combined comprised up to 25% of the weight of the $Et_2O$ extract). Two visits to the Catalina Marine Science Center were made, in June and July 1987 and March 1990.

The results from the 1987 feeding experiments appear in Table 1. This work demonstrated for the first time that brominated Verongida metabolites **1** and **2** are indeed derivable from 3,5-$Br_2$Tyr [**4**]. Tyr is incorporated into **1**, as previously reported, but this is the first report of its being incorporated into **2**. 3-BromoTyr [**5**] was also incorporated into both **1** and **2** relatively well, but neither 3-bromo-4-hydroxybenzyl cyanide [**10**] nor 3,5-dibromo-4-hydroxybenzyl cyanide [**8**] was significantly incorporated into **1** or **2** in any of the feeding experiments.

The amount of healthy sponge accessible at the Catalina Marine Science Center was severely limited during the 1990 field work. Several small specimens of *A. fistularis* were surveyed for brominated tyrosine metabolites on Catalina Island by grinding sponge tissue in MeOH in a mortar and pestle. Tlc [$CHCl_3$-MeOH (9:1)] performed immediately on the MeOH extract revealed that **2** was present but that the other major metabolite, **1**, was not detectable. After bringing the frozen sponges used in the feeding experiments back to the University of Illinois and extracting for 2 days with $Et_2O$ in a Soxhlet extractor, however, all sponge samples contained sizeable amounts of **1**.

The uptake of radioactivity was measured at various times during the two-day incubation period for all the $^{14}C$ feeding experiments except for 4-methoxybenzyl [$^{14}C$]cyanide [**17**]. The amount of radioactivity in the aquarium sea water significantly decreased in all experiments. The uptake of [*methyl*-$^{14}C$]Met was quite dramatic, presumably due to the high specific activity of this precursor. The incorporation results of the March 1990 feeding experiments are summarized in Table 2.

Tyr was again shown to be incorporated into **2** by the carbon-14 results, but the incorporation level was too low to detect any $^{13}C$ enrichment by isotope ratio mass spectrometry or nmr. The low incorporation compared to the 1987 results is presumably due to the high dilution of label from adding the large amount of $^{13}C$-labeled Tyr to the high specific activity $^{14}C$-labeled Tyr.

The incorporation of [*methyl*-$^{14}C$]Met into the lipid fractions was relatively high (0.09%), as might be expected, and incorporation into **2** was low but significant. Compound **1** was not significantly labeled, as would be predicted. To demonstrate that Met specifically labeled **2** at the *O*-methyl group, **2** was converted to dienone **34** using a combination of synthetic conversions described by Capon (Scheme 6) (23); as expected, the purified product was not radioactive.

The three $^{14}C$-labeled *O*-methyl nitriles **12**, **17**, and **18** were not incorporated into **1** or **2**. For the isolation of metabolites from these three feeding experiments, the $Et_2O$ extracts were each spiked with 20 mg of cold precursor and each was isolated in early fractions of the Si gel gravity column used for isolation of the metabolites. Each isolated

TABLE 1. Incorporation of $^{14}C$-Labeled Potential Precursors (1987 Collection).

| Precursor | Tyr | **10** | **8** | BrTyr [**5**] | $Br_2$Tyr [**4**] | **10** | **8** | $Br_2$Tyr [**4**] | **10** | **8** |
|---|---|---|---|---|---|---|---|---|---|---|
| DPM$\times 10^8$ | 1.11[a] | 1.07 | 1.23 | 1.02 | 1.10 | 1.07 | 4.48 | 1.14 | 1.08 | 4.44 |
| DPM/mmol$\times 10^9$ | 4.02 | 10.8 | 1.68 | 9.08 | 12.9 | 10.8 | 57.4 | 13.3 | 10.4 | 56.7 |
| Experiment type | 2 days closed 1 week open | 2 days closed 1 week open | | 2 days closed 3 weeks open | 2 days closed 3 weeks open | 2 days closed 3 weeks open | | 3 days closed | 3 days closed | |
| Aeroplysinin-1 [**2**] | | | | | | | | | | |
| DPM | 80,400 | 0 | 0 | 89,800 | 59,600 | 0 | 0 | 4,200 | 0 | 0 |
| DPM/mmol$\times 10^5$ | 23 | | | 2.7 | 1.9 | | | 0.34 | | |
| % incorporation$\times 10^{-2}$ | 7.2 | | | 8.8 | 5.4 | | | 0.37 | | |
| dilution of $^{14}C\times 10^3$ | 1.7 | 0 | 0 | 36 | 67 | 0 | 0 | 39 | 0 | 0 |
| Dibromoverongiaquinol [**4**] | | | | | | | | | | |
| DPM | 556,000 | 0 | 0 | 135,000 | 80,600 | 0 | 0 | 32,000 | 0 | 0 |
| DPM/mmol$\times 10^5$ | 25 | | | 2.7 | 1.7 | | | 0.76 | | |
| % incorporation$\times 10^{-2}$ | 50 | | | 13 | 7.3 | | | 2.9 | | |
| dilution of $^{14}C\times 10^3$ | 1.6 | | | 36 | 76 | | | 15 | | |

[a]Numbers should be multiplied by the exponents shown in the first column.

TABLE 2. Incorporation of $^{14}C$-Labeled Potential Precursors (1990 Collection).[a]

| | Tyr | MeTyr [25] | $MeBr_2Tyr$ [26] | Met | 17 | 12 | 18 |
|---|---|---|---|---|---|---|---|
| Precursor | | | | | | | |
| DPM×$10^8$ | 1.11[b] | 1.02 | 1.14 | 1.11 | 2.78 | 0.955 | 1.98 |
| DPM/mmol×$10^9$ | 0.229 | 4.35 | 8.04 | 124 | 1.58 | 1.07 | 5.74 |
| Aeroplysinin-1 [2] | | | | | | | |
| DPM | 14,400 | 0 | 0 | 6,060 | 0 | 0 | 0 |
| DPM/mmol×$10^5$ | 4.2 | | | 1.6 | | | |
| % incorporation×$10^{-2}$ | 1.3 | | | 0.55 | | | |
| dilution of $^{14}C$×$10^3$ | 5.5 | | | 74,000 | | | |
| Sponge tissue | | | | | | | |
| DPM×$10^7$ | 1.2 | 3.7[c] | 1.2[d] | 1.2 | 1.1 | 0.17 | 0.48 |
| % incorporation | 11 | 23 | 6.3 | 11 | 3.9 | 1.8 | 2.4 |

[a]All experiments were 2 days closed, 1 week open.
[b]Numbers should be multiplied by the exponents shown in the first column.
[c]From 1.02 DPM×$10^8$ labeled **25**.
[d]From 1.14 DPM×$10^8$ labeled **26**.

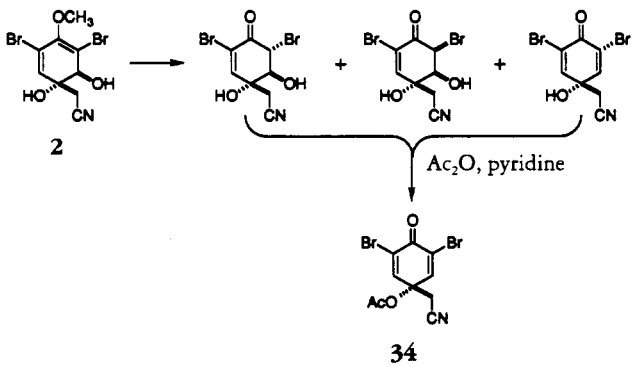
precursor was then purified by prep. hplc [hexane-EtOAc (85:15)], and none of the precursors contained any radioactivity.

Neither of the $^{14}C$-labeled *O*-methyl tyrosines, **25** or **26**, was incorporated into **1** or **2**.

Portions of whole sponge tissue were digested with Protosol and counted, and it was found, surprisingly, that incorporations of up to 23% of the labeled precursors into the tissue had occurred (Table 2).

That Tyr, $Br_2Tyr$ [**4**], and BrTyr [**5**] were incorporated into dibromoverongiaquinol [**1**] in the 1987 feedings supports the biosynthetic pathway in Scheme 1. Comparison of the three-day feeding experiment with **4** to the two-day closed, three-week open experiment with **4** shows that incorporation of **4** into both **1** and **2** was slightly greater for the longer cultivation period. In contrast to expectations, Tyr was incorporated more efficiently than BrTyr [**5**], which in turn was incorporated more efficiently than $Br_2Tyr$ [**4**]. This may result from the bulky bromines interfering with the permeability of the precursors across cell membranes.

It was surprising that neither nitrile **8** nor **10** (di- or mono-bromo-*p*-hydroxybenzyl cyanide) was significantly incorporated into **1** or **2** in any of the six feeding experiments. Both of these nitriles have been detected as metabolites of *A. fistularis* (24) and their

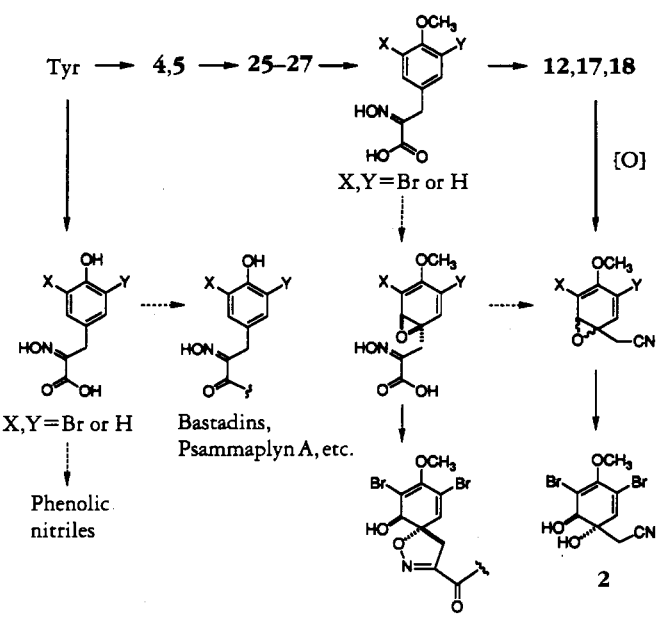



SCHEME 6

involvement as precursors to **1** as depicted in Scheme 1 seems reasonable. The possible reasons for their not being incorporated into **1** and **2**, aside from the possibility of their not being on the biosynthetic path, are poor transport of the halogenated nitriles across cell membranes or poor solubility of the nitriles in sea water. Since neither **8** nor **10** was incorporated into **1** or **2**, no information about the ability of the haloperoxidase enzyme(s) involved in the bromination of advanced precursors can be inferred, but the enzyme(s) can apparently brominate Tyr and BrTyr.

Goo has suggested that the verongiaquinol and homogentisic acid derivatives are biosynthesized from an arene oxide derived only from the corresponding hydroxy metabolites of Tyr, but not from their methoxy analogues, and that an arene oxide from an *O*-methylated Tyr intermediate would be metabolized by nucleophilic opening of the epoxide to lead to **2** (24,25). Alternatively, and with the 1987 results in mind, *O*-methylation could occur prior to formation of the nitrile functional group, and thus the phenolic nitrile would not be a suitable precursor to **2**. In general, *O*-methylation could occur relatively early in the biosynthetic pathway, with the *O*-methyl nitrile serving as the precursor to both **1** and **2** (Scheme 7). Recent results from another laboratory, demonstrating that **2** can be converted to **1** with base or a crude enzyme preparation from *A. aerophoba* (personal communication, Prof. Peter Proksch, Universität Würzburg), supports this proposed pathway. It is noteworthy that all Verongida metabolites with isoxazoline rings also have *O*-methyl groups, but several of the highly elaborated metabolites such as the bastadins and psammaplyn A contain α-oximino amides and have free phenol groups. It was thus proposed that the *O*-methyl group is required for the formation of the arene oxide intermediate which leads to **1**, **2**, and the isoxazoline metabolites, and metabolites with free phenols are converted to α-oximino compounds or to the phenolic nitriles. Several labeled compounds were prepared to test this hypothesis.

In the 1990 feedings, $^{14}$C-labeled Tyr and Met were both incorporated into aeroplysinin-1 [**2**]. Although the Met incorporation was low and with a large dilution, these findings are in contrast to early work where Met was not incorporated at all into




SCHEME 7

**2** or aerothionin by *A. aerophoba* (7). These conflicting results are presumably due to the longer culture times used in the present study. Although the incorporation in the Tyr experiment was too low for useful results with $^{13}C$-labeled precursors, the results from the experiment with $^{14}C$ label demonstrate that the sponge was producing the metabolites during this time of year.

None of the more advanced potential precursors was incorporated into **1** or **2**. All the precursors were apparently taken up during the 2-day incubation period, with the extent of uptake for all precursors except one being greater than that for Tyr, which was incorporated into **2**. Since the precursors were apparently taken up but not converted into the halogenated metabolites, it was surprising that no labeled nitrile precursors were recovered during the isolation. In fact, the radioactivities of the $Et_2O$ extracts for some of the experiments were measured and all were below 50,000 dpm, corresponding to an incorporation at this point of less than 0.05%. It could be possible that the precursors were taken up during the incubation period and then exuded gradually as different metabolites while fresh sea water was passing through the aquarium. Aerothionin has been shown to be exuded from *A. fistularis* (26), but with such a large pool of **2**, it is unlikely that there is a 100% turnover of **2** in the sponge during the incubation period.

The search for the fate of the precursors led to digestion of the extracted sponge tissue. An intriguing result from these experiments was the high amount of carbon-14 remaining in the sponge tissue. Halogenated tyrosines have been found in sponge scleroprotein (27), but it is not likely that the nitriles could be entirely converted to the amino acids in the protein. It is interesting that in Bergquist's survey of free amino acids in Demospongiae, no Tyr was detected in *A. fistularis* (28).

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined on either a Reichert hot stage or a Thomas-Hoover capillary apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Elemental analyses were determined by the University of Illinois Microanalytical Lab.

DMF was distilled at reduced pressure from BaO; $C_6H_6$, $Et_2O$, and $CH_2Cl_2$ and $C_5H_5N$ were distilled under $N_2$ from $CaH_2$, $LiAlH_4$, $P_2O_5$ and KOH, respectively. *p*-Hydroxybenzyl alcohol (Aldrich) was recrystallized from $H_2O$ immediately prior to use. NBS was recrystallized from $H_2O$ and dried under vacuum over $P_2O_5$. $K_2CO_3$ was pulverized with a mortar and pestle and dried in an Abderhalden pistol at 110° overnight. KCN was purified as described (29) immediately prior to use. Trifluoroacetic anhydride was distilled from $P_2O_5$ immediately prior to use. Carboxypeptidase A, Type II-DFP (54 units/mg, 16.9 mg/ml) was purchased from Sigma. Sodium [$^{14}C$]cyanide was purchased from ICN Radiochemicals (Irvine, CA). Sodium [$^{13}C$]cyanide was provided (in 1974) by the Los Alamos Stable Isotope Resource. L-[*U*-$^{14}C$]Tyrosine was obtained from Amersham Corporation (Arlington Heights, IL) and ICN, and [*methyl*-$^{14}C$]methionine was obtained from Amersham. [$^{14}C$]Methyl and [$^{13}C$]methyl iodides were purchased from Amersham and Aldrich, respectively. All other chemicals were reagent grade and used without further purification.

Ir spectra were recorded on a Perkin-Elmer 1320 or a Nicolet MX-5 spectrophotometer and were referenced to the 1601 $cm^{-1}$ band of polystyrene. $^1H$- and $^{13}C$-nmr spectra were measured on Varian XL-200 and General Electric QE-300 instruments. Chemical shifts are reported in parts per million (δ) downfield from TMS or sodium 3-(trimethylsilyl)propionate. Low-resolution eims were obtained with a Finnigan MAT CH5 DF spectrometer.

Tlc was performed on precoated Si gel plates (Kieselgel $60F_{254}$, Merck, layer thickness 0.25 mm). The usual tlc developing solvents were $CHCl_3$-MeOH (9:1 or 4:1) and $C_6H_6$-EtOAc (5:1) for nitriles and sponge metabolites and EtOAc-$C_5H_5N$-HOAc-$H_2O$ (30:20:6:11) and *n*-BuOH-$H_2O$-HOAc (4:1:1, 2×) for amino acids. Amino acids were purified by eluting Sephadex G-15 (Pharmacia Fine Chemicals, particle size 40–120 μm) with 0.02 N HOAc.

Hplc was performed on a system comprised of Beckman 114M pumps, a Rheodyne injector, and a Beckman 153 8-μl analytical uv detector at 254 nm fixed wavelength. Alltech semipreparative silica, cyano, and $C_{18}$ columns (1×25 cm, 10 μm particle size) were used at a flow rate of 5 ml/min.

Chiral gas chromatographic analyses were carried out with a Varian 3700 gas chromatograph and a

flame ionization detector using a Chirasil Val-III column (0.35 mm×25 m) and a temperature program of 90° for 4 min, then 4° per min to 180°.

RADIOISOTOPE MEASUREMENTS.—Small aliquots of radiolabeled samples (usually 1/32,000 for precursors, 1/20 for isolated metabolites) were dissolved in 15 ml of Aquasol or PPO/toluene scintillant and the radioactivity was measured with a Tracor Analytic Betatrac 6895 liquid scintillation counter.

The radiochemical purity of precursors was assessed on a Radiomatic Instruments and Chemical Co., Inc. rtlc scanner using Si gel tlc plates developed in at least two solvent systems. Each plate had two sets of lanes of labeled compound, the labeled compound co-spotted with authentic unlabeled material, and unlabeled material. The metabolites isolated were either chromatographed repeatedly by hplc using several solvent systems or recrystallized to constant specific activity.

Whole sponge tissue from the 1990 experiments was counted by pulverizing sponge tissue with a mortar and pestle and placing approximately 100 mg in a scintillation vial. The tissue was moistened with 0.4 ml of $H_2O$, 1 ml of Protosol was added, and the mixture was stirred overnight. To the mixture was added 100 μl of 30% $H_2O_2$, the mixture was stirred an additional 1 h and 15 ml of Aquasol was added, followed by 100 μl of glacial HOAc. Counting was done typically 3–4 h after the addition of HOAc, and all samples were counted again 12 h later. Sponge tissue from $^{13}C$-labeled feeding experiments was digested side-by-side in the same manner to serve as background reference.

The uptake of radioactivity in the March 1990 experiments was measured at Catalina Marine Science Center (CMSC) on a Beckman LS1801 liquid scintillation counter by dissolving a 1.0-ml aliquot of aquarium sea water in 15 ml of Ready Solv MP cocktail. No quench curves or counting efficiencies were determined, but the instrument was calibrated against an external reference.

ANIMAL MATERIAL.—*Aplysina fistularis* Pallas (1766) (synonyms *Verongia thiona, V. aurea, V. tenuissima, V. fistularis, Spongia fulva, S. fistularis*) (Aplysinidae) was collected at South Headland Cave, Big Fisherman's Cove, Catalina Island, California, at a depth of 0–5 m, by chipping away sections of the rock substrate with the sponge intact. The specimens were placed in a bucket of ambient sea water and immediately transported to the Catalina Marine Science Center laboratory without exposure to air. For each experiment, sponges (30–75 g squeezed dry wt) were placed in a 1-gallon glass jar containing 3 liters of unfiltered, continuously aerated sea water, maintained in the dark and at environmental temperature by a fresh running sea water bath surrounding the jar. An antibiotic mixture of 0.5 g penicillin G, 0.2 g streptomycin sulfate, and 0.4 g chloramphenicol was then added to the jar to prevent bacterial infection. After 2–3 h, a continuous flow of sea water was added to the jar, and the sponges were left overnight.

FEEDING EXPERIMENTS.—The continuous flow of sea water to the jar was stopped and the radiolabeled precursor, dissolved in 1 ml of MeOH (nitriles) or 1 ml of 1 N HCl (amino acids), was added in one portion. In some runs sponges were maintained for 3 days and the experiment was terminated; in others, after 2 days a continuous flow of sea water was added to the jar for 7 or 21 days and the experiment was then terminated (see Table 1). The Tyr and 3-week $Br_2$Tyr experiments were initiated on June 15 and June 29, 1987, respectively, and the other summer 1987 experiments were initiated on July 11, 1987. The 1990 tyrosine and methionine experiments were initiated on March 4, 1990, and the remaining 1990 experiments were started on March 1, 1990.

EXTRACTION AND ISOLATION.—In 1987, the sponges were transported alive to the University of Southern California main campus in Los Angeles, where they were cut up, squeezed dry and weighed. The tissue was extracted for 2 days with 500 ml of $Et_2O$ in a Soxhlet apparatus. The extracts were dried over $MgSO_4$, concentrated *in vacuo*, and shipped to the University of Illinois, Urbana, on dry ice. Each extract was then chromatographed on 40× its weight of Si gel (Merck Kieselgel 60, 230–400 mesh), eluting initially with $Me_2CO$-$C_6H_6$ (15:85). After elution of aeroplysinin-1 [**2**] the solvent was changed to $Me_2CO$-$C_6H_6$ (30:70) to elute dibromoverongiaquinol [**1**].

*Dibromoverongiaquinol* [**1**].—Dibromoverongiaquinol was purified by $C_{18}$ reversed-phase hplc [$H_2O$-MeOH (2:1) or $H_2O$-MeCN (8:2)] and by recrystallization from MeCN, $H_2O$, or $CH_2Cl_2$: mp 192–193° (dec) [lit. (1) 193–195° (dec)].

*Aeroplysinin-1* [**2**].—Aeroplysinin-1 was purified by $C_{18}$ reversed-phase hplc [$H_2O$-MeOH (2:1)] and by recrystallization from $CHCl_3$: $[\alpha]^{25}D$ +176.4° ($c$=0.181, MeOH) [lit. (3) rotation for this enantiomer+186° (MeOH)]; mp 117–118° (lit. (3) 120–121°).

In 1990, sponges were blotted dry and frozen on Catalina Island in a −70° freezer and brought back to the University of Illinois, Urbana, in an ice chest containing blue ice. The metabolites were then isolated as described above.

SYNTHESIS OF PRECURSORS AND OTHER REACTIONS.—*Preparation of 2-(1'-acetoxy-3',5'-dibromo-4'-oxocyclohexa-2',5'-dienyl)-acetonitrile* [**34**] (23).—A 30-mg sample of **2** from the [*methyl*-$^{14}CH_3$]methionine

feeding experiment was treated with 1 ml of trifluoroacetic acid for 30 min. The trifluoroacetic acid was removed with a stream of $N_2$, the residue was taken up in 1 ml of dry pyridine, and 200 μl of $Ac_2O$ was added. The mixture was stirred for 2 days and then concentrated with a stream of $N_2$. The residue was purified by Si gel cc [$C_6H_6$-$Me_2CO$ (95:5)] to give 20 mg (65%) of **34**, which crystallized upon concentration from $CHCl_3$: mp 109–111° [lit. (23) reported as an oil]; $^1H$ nmr ($CDCl_3$) δ 2.15 (3H, s, $COCH_3$), 2.96 (2H, s, $CH_2$), 7.38 (2H, s, H-2′, H-6′); $^{13}C$ nmr ($CDCl_3$) δ 20.9 ($CH_3$), 28.3 ($CH_2$), 75.0 (C-1′), 113.2 (C-3′, C-5′), 124.9 (CN), 144.4 (C-2′, C-6′), 168.6 (*C*O$CH_3$), 171.1 (C-4′).

[U-$^{14}C$]-L-*3,5-Dibromotyrosine* [**4**].—[*U*-$^{14}C$]-L-Tyr (99.7 μCi, specific activity 522 mCi/mmol) in 1 N HCl was lyophilized to dryness and 3.00 mg (16.6 μmol) of cold L-Tyr and 1.0 ml of HOAc were added. To the stirred suspension was added a solution of 2.1 μl (41 μmol) of $Br_2$ in 1.0 ml HOAc. After 6 h stirring at 60°, rtlc showed neither Tyr nor 3-BrTyr was present. Lyophilization afforded 5.69 mg, 99.7 μCi of labeled **4** (100% radiochemical yield).

[U-$^{14}C$]-L-*3-Bromotyrosine* [**5**].—[*U*-$^{14}C$]-L-Tyr (50.1 μCi) in 1 N HCl was lyophilized to dryness. The residue was dissolved in 20 ml of 0.5 N HCl and 6.90 mg (38.1 μmol) of carrier Tyr was added. To the stirred solution was added a solution of 2.23 mg (13.3 μmol) of $KBrO_3$ and 9.52 mg (80.0 μmol) of KBr in 20 ml of $H_2O$. When rtlc showed that no Tyr remained, the mixture was lyophilized to dryness. The residue was dissolved in 0.5 ml of 0.02 N HOAc and chromatographed on a Sephadex G-15 column (1.0×17.0 cm), eluting with 0.02 N HOAc to give 9.94 mg (46 μCi, 92% radiochemical yield) of labeled **5**.

*3,5-Dibromo-4-hydroxybenzyl bromide* [**9**].—To a stirred solution of 4.6 g (37 mmol) of 4-hydroxybenzyl alcohol in 100 ml of HOAc was added 4.4 ml (86 mmol) of $Br_2$ in 50 ml of HOAc. After being stirred for 20 h the mixture was cooled in an ice bath and the precipitate was collected and washed with a small portion of cold HOAc to afford 8.1 g (63%) of **9** as a white powder, mp 149–150°. The mother liquor was concentrated to give a yellow solid which was washed with a small portion of cold HOAc. Recrystallization from $C_6H_6$ gave an additional 2.0 g (16%) of **9**: mp 149–150° [lit. (30) 150°].

*3,5-Dibromo-4-hydroxybenzyl* [$^{14}C$]*cyanide* [**8**].—A solution of 13.2 mg (38.3 μmol) of **9** in 0.5 ml of DMF was added to a screw-capped vial containing 1.1 mg (1.0 mCi) of $Na^{14}CN$ (specific activity 58.2 mCi/mmol) and a stirring bar. After being stirred sealed for 2 h at room temperature, 1.1 mg (22.4 μmol) of carrier NaCN was added, the vial was sealed, and the mixture was stirred an additional 40 h. The mixture was filtered and purified by prep. hplc [cyano column, MeOH-$H_2O$-HOAc (30:70:1)] to afford 6.60 ~~mg~~ (610 μCi, radiochemical yield 61%) of labeled **8**.

*2-Bromo-4-methylanisole.*—A solution of 7.3 g of NBS (41 mmol) and 5.0 g (41 mmol) of 4-methylanisole in 100 ml of DMF was stirred at room temperature for 26 h. The mixture was poured into 500 ml of $H_2O$ and extracted with $CH_2Cl_2$ (2×350 ml). The organic layer was washed with $H_2O$ (2×300 ml) and brine (200 ml), and dried over $MgSO_4$ to give 7.4 g of crude bromide. Si gel cc [hexane-$Et_2O$ (15:1)] gave 6.7 g (82%) of pure 2-bromo-4-methylanisole: bp 234–236° [lit. (38) 227.5°].

*3-Bromo-4-methoxybenzyl bromide* [**11**].—A mixture of 5.0 g (25 mmol) of 2-bromo-4-methylanisole, 4.6 g (26 mmol) of NBS, and 70 mg (0.29 mmol) of benzoyl peroxide in 50 ml of $CCl_4$ was heated at reflux under $N_2$ for 20 h and then filtered to remove succinimide. The filtrate was washed with 20 ml of $H_2O$, dried over $MgSO_4$, and concentrated *in vacuo*. Recrystallization from low-boiling petroleum ether gave 4.2 g (60%) of **11**: mp 62–63° [lit. (24) 61–62°].

*3-Bromo-4-methoxybenzyl* [$^{14}C$]*cyanide* [**12**].—To 0.28 mg of $Na^{14}CN$ (250 μCi, specific activity 58.4 mCi/mmol) and 2.40 mg (49 μmol) of carrier NaCN in a screw-capped vial containing a stirring bar was added a solution of 14.0 mg (50 μmol) of **11** in 0.5 ml of DMF. The vial was sealed and the mixture was stirred at 110° for 24 h. After it had cooled, the mixture was filtered and purified by prep. hplc [Si gel, hexane-EtOAc (7:3)] to give 9.72 mg (210 μCi, radiochemical yield 84%) of labeled **12**.

*3-Bromo-4-hydroxybenzyl* [$^{14}C$]*cyanide* [**10**].—To a solution of the entire sample of labeled **12** in 0.5 ml of $CH_2Cl_2$ in a screw-capped vial was added 160 μl of 1.0 M $BBr_3$ in $CH_2Cl_2$. After 6 h, rtlc revealed the reaction was complete. To the vial was added 0.5 ml of ice-cold $H_2O$, the layers were separated, and the aqueous layer was extracted with 0.5 ml of $CH_2Cl_2$ (3×). The combined organic layers were concentrated and purified by prep. hplc [cyano, MeOH-$H_2O$-HOAc (30:70:1)] to give 8.75 mg (202 μCi, radiochemical yield 96%) of labeled **10**.

*4-Methoxybenzyl* [$^{14}C$]*cyanide* [**17**].—A solution of 27.3 mg (174 μmol) of fresh 4-methoxybenzyl chloride and 49.1 mg (186 μmol) of 18-crown-6 in 250 ml of dry MeCN was added to 1 mCi (1.19 mg, specific activity 56.2 mCi/mmol) of $K^{14}CN$ in the vial the material was supplied in. After 6 h stirring at room temperature, 8.40 mg (129 μmol) of carrier KCN was added and the mixture was stirred overnight. The mixture was concentrated *in vacuo*, taken up in a small amount of EtOAc, and passed through a Sep Pak column, eluting with EtOAc. Prep. hplc [Si gel, hexane-EtOAc (85:15)] gave 21.08 mg, 1.02 mCi (102% radiochemical yield) of radiochemically pure **17**.

*3,5-Dibromo-4-methoxybenzyl [$^{14}C$]cyanide* [**18**].—Excess $CH_2N_2$ in $Et_2O$ was added to a solution of 178 μCi of 3,5-dibromo-4-hydroxybenzyl [$^{14}C$]cyanide [**8**] in 200 μl of $Et_2O$ in a 1-dram vial, and the vial was sealed and left overnight. Rtlc using several solvent systems showed no starting material was present and that the material was radiochromatographically pure **18**: 178 μCi (100% radiochemical yield).

*3-Bromo-4-methoxybenzyl [$^{14}C$]cyanide* [**12**].—Excess $CH_2N_2$ was added to a 1-dram vial containing a solution of 49 μCi of **10** in 200 μl of $Et_2O$. The vial was sealed and left overnight. Rtlc analysis showed that no starting material remained, but several minor by-products were present. The mixture was purified by prep. hplc [Si gel, hexane-EtOAc (8:2)] to afford 43 μCi (88% radiochemical yield) of radiochemically pure **12**.

N-(t-*Butyloxycarbonyl*)-L-*tyrosine* t-*butyl ester* [**22**].—To a solution of 0.500 g (2.11 mmol) of L-tyrosine *t*-butyl ester in 10 ml of $Et_2O$ and 10 ml of *t*-butyl alcohol was added 0.48 ml (2.09 mmol) of di-*t*-butyl dicarbonate. The solution refluxed for 5 h and was concentrated *in vacuo* to give a white powder. Si gel cc [$C_6H_6$-EtOAc (5:1)] gave 0.681 g (96%) of **22**: mp 115–116°; $[\alpha]^{21}D$ +37.2° (*c*=0.290, $CHCl_3$); ir ν max (neat) 3442, 2996, 2937, 1741, 1689, 1511, 1379, 1233, 1155 $cm^{-1}$; $^1H$ nmr ($CDCl_3$) δ 1.42 [9H, s, $C(CH_3)_3$], 1.62 [9H, s, $C(CH_3)_3$], 2.97 (2H, br d, *J*=5.7 Hz, H-β), 4.37 (1H, dt, *J*=5.7 and 7.9 Hz, H-α), 5.03 (1H, d, *J*=7.9 Hz, NH), 6.81 (2H, d, *J*=8.5 Hz, H-3, H-5), 7.08 (2H, d, *J*=8.5 Hz, H-2, H-6); $^{13}C$ nmr ($CDCl_3$) δ 28.1 [-$C(CH_3)_3$], 28.5 [-$C(CH_3)_3$], 37.8 (H-β), 55.2 (H-α), 80.2 [-*O*$C(CH_3)_3$], 82.4 [-*O*$C(CH_3)_3$], 115.5 (C-3, C-5), 128.5 (C-2, C-6), 130.7 (C-1), 155.3 (C-4), 155.5 (-NHCOO-), 171.5 (-COO-); *anal.*, calcd for $C_{18}H_{27}NO_5$, C, 64.07, H, 8.07, N, 4.15; found C, 64.08, H, 8.11, N, 4.21.

N-(t-*Butyloxycarbonyl*)-L-*3-bromotyrosine* t-*butyl ester* [**24**].—The first portion of 0.55 g (3.1 mmol) of NBS in 10 ml of DMF was added dropwise to a solution of 2.00 g (5.93 mmol) of **22** in 9 ml of dry DMF. After addition was complete, the second portion of 0.55 g (3.1 mmol) of NBS in 10 ml of DMF was added dropwise, and the solution was stirred overnight. The reaction mixture was poured into 300 ml of $H_2O$ and extracted with $CH_2Cl_2$ (200 ml×2). The combined organic layers were washed with 200 ml of brine, dried over $Na_2SO_4$, and concentrated. Si gel cc [$C_6H_6$-EtOAc (6:1)] gave 1.53 g (62%) of **24**: $[\alpha]^{21}D$ +43.8° (*c*=0.894, $CHCl_3$); mp 159.5–160°, $^1H$ nmr ($CDCl_3$) δ 1.41 [9H, s, $C(CH_3)_3$], 1.43 [9H, s, $C(CH_3)_3$], 2.98 (2H, complex, H-β), 4.38 (1H, *J*=7.8 and 5.7 Hz, H-α), 5.01 (1H, d, *J*=7.8 Hz, -NH-), 5.43 (1H, s, -OH), 6.92 (1H, d, *J*=8.3 Hz, H-5), 7.03 (1H, dd, *J*=8.3 and 1.8 Hz, H-6), 7.25 (1H, br s, H-2); $^{13}C$ nmr ($CDCl_3$) δ 28.0 [-$C(CH_3)_3$], 28.3 [-$C(CH_3)_3$], 37.2 (C-β), 54.9 (C-α), 79.9 [-*O*$C(CH_3)_3$], 82.4 [-*O*$C(CH_3)_3$], 109.8 (C-3), 115.9 (C-5), 129.9 (C-2), 130.2 (C-6), 133.0 (C-1), 151.4 (C-4), 155.1 (-NHCOO-), 170.7 (-COO-).

[Methyl-$^{13}C$]-L-*3-bromo*-O-*methyltyrosine* [**27**].—To a stirred ice-cold mixture of 0.706 g (1.70 mmol) of **24**, 0.235 g (1.70 mmol) of $K_2CO_3$, and 0.461 g (1.74 mmol) of 18-crown-6 in 25 ml of dry $C_6H_6$ was added 150 μl (2.39 mmol) of [$^{13}C$]methyl iodide. After being stirred for 6 h the mixture was poured into 20 ml of $H_2O$, the layers were separated, and the organic layer was washed with brine (2×15 ml), dried over $Na_2SO_4$, and concentrated *in vacuo*. The product was taken up in 10 ml of $CH_2Cl_2$ and 10 ml of trifluoroacetic acid was added. The mixture was stirred overnight, concentrated, and taken up in $H_2O$. The pH was adjusted to 6.0 (pH meter) with $NH_4OH$ and the precipitate was collected and dried in an Abderhalden pistol (110°) overnight to give 0.439 g (94%) of **27**: mp 220–227° (dec); $[\alpha]^{22}D$ +0.05° (*c*=4.78, 1 N HCl); $^1H$ nmr ($D_2O$, DCl) δ 3.03 (1H, dd, *J*=15.1 and 7.6 Hz, H-β), 3.16 (1H, dd, *J*=15.1 and 5.6 Hz, H-β), 3.80 (3H, d, $J_{(C\text{-}H)}$=146.0 Hz, -$CH_3$), 4.10 (1H, dd, *J*=7.3 and 5.8 Hz, H-α), 6.99 (1H, d, *J*=8.4 Hz, H-5), 7.18 (1H, dd, *J*=8.4 and 1.9 Hz, H-6), 7.43 (1H, d, *J*=1.9 Hz, H-2); $^{13}C$ nmr ($D_2O$, DCl) δ 56.2 (-$CH_3$).

[Methyl-$^{13}C$]-L-O-*methyltyrosine* [**25**].—To an ice-cold mixture of 0.700 g (2.07 mmol) of **22**, 0.316 g (2.29 mmol) of $K_2CO_3$, and 0.560 g (2.12 mmol) of 18-crown-6 in 25 ml of dry $C_6H_6$, was added 105 μl (1.67 mmol) of [$^{13}C$]methyl iodide. After being stirred for 24 h at room temperature, the mixture was poured into 20 ml of $H_2O$ and the organic layer was washed with 10 ml of 2 N NaOH followed by brine (2×15 ml), dried ($Na_2SO_4$), and concentrated *in vacuo* to give 0.618 g of solid. The product was dissolved in 10 ml of $CH_2Cl_2$ and 10 ml of trifluoroacetic acid was added. After being stirred overnight the mixture was concentrated, taken up in $H_2O$, and adjusted to pH 6.0 (pH meter) with $NH_4OH$. The precipitate was dried overnight (Abderhalden pistol, 110°) to give 0.172 g of **25**. Partial concentration of the mother liquor gave an additional 0.113 g of **25** (86% total yield based on $^{13}CH_3I$): $[\alpha]^{22}D$ −6.9° (*c*=0.348, 1 N HCl) [lit. (43) for unlabeled compound −5.7° (*c*=2.02, 3 N HCl)]; $^1H$ nmr ($D_2O$, DCl) δ 3.32 (1H, dd, *J*=14.7 and 7.5 Hz, H-β), 3.44 (1H, dd, *J*=14.7 and 5.6 Hz, H-β), 3.96 (3H, d, $J_{(C\text{-}H)}$=145.2 Hz, -$CH_3$), 4.49 (dd, *J*=7.2 and 5.7 Hz, H-α), 7.14 (2H, d, *J*=8.6 Hz, H-3, H-5), 7.41 (2H, d, *J*=8.6 Hz, H-2, H-6); $^{13}C$ nmr ($D_2O$, DCl) δ 58.1 (-$CH_3$).

[Methyl-$^{14}C$]-L-O-*methyltyrosine*.—The break-seal ampoule that 100 μCi of [$^{14}C$]methyl iodide (54 mCi/mmol) was supplied in was cooled in a dry ice/*i*-PrOH bath. An ice-cooled mixture of 12.75 mg (37.79 mmol) of **22**, 6.95 mg (50.2 μmol) of $K_2CO_3$, and 15.55 mg (58.83 μmol) of 18-crown-6 in 1 ml of dry

$C_6H_6$ was added to the ampoule, the ampoule was sealed, and the mixture was stirred at room temperature for 2 days. To the ampoule was added 200 μl of unlabeled methyl iodide, and the mixture was stirred an additional day. The reaction was poured into 1 ml of $H_2O$, the layers were separated, and the organic layer was stirred with 0.5 ml of 2 N NaOH for 1 h. The organic layer was washed with 0.5 ml of brine and concentrated *in vacuo*. The residue was taken up in 300 μl of trifluoroacetic acid, the vial was sealed, and the mixture was stirred overnight. Rtlc analysis showed that the reaction was complete. The mixture was concentrated with a stream of $N_2$ to give 74 μCi (74%) of [*methyl*-$^{14}$C]-L-*O*-methyltyrosine.

L-*3,5-Dibromotyrosine t-butyl ester*.—Liquified isobutylene (30 ml) was added to a solution of 6.87 g (20.0 mmol) of L-dibromotyrosine [**4**] and 7.60 g (40 mmol) of *p*-toluenesulfonic acid monohydrate in 30 ml of dioxane in a pressure bottle cooled with dry ice. The mixture was shaken on a Parr apparatus for 24 h and poured into an ice-cold mixture of 120 ml of EtOAc and 120 ml of 0.25 N NaOH. The pH was adjusted to 9 (pH paper) with 10% citric acid and the ester was extracted into EtOAc (2×200 ml). The combined organic layers were concentrated to give a brown powder which was recrystallized from EtOAc/hexane to give 3.79 g (48%) of the ester: mp 161.5–163°; $[\alpha]^{21}$D +22.8° (*c*=0.499, MeOH); ir (Nujol) ν max 3325, 3227, 1736, 1294, 1157, 834, 730 cm$^{-1}$; $^1$H nmr ($CDCl_3$) δ 1.38 [9H, s, -C($CH_3$)$_3$], 2.77 (1H, dd, *J*=13.8 and 7.3 Hz, H-β), 2.85 (1H, dd, *J*=13.8 and 6.2 Hz, H-β), 3.57 (1H, br t, *J*=6.6 Hz, H-α), 7.29 (2H, s, H-2, H-6); $^{13}$C nmr ($CDCl_3$) δ 28.0 [-C($CH_3$)$_3$], 39.2 (C-β), 56.0 (C-α), 81.8 [-*O*C($CH_3$)$_3$], 110.2 (C-3, C-5), 131.7 (C-2, C-6), 132.8 (C-1), 148.5 (C-4), 173.8 (-COO-); *anal*., calcd for $C_{13}H_{17}Br_2NO_3$, C, 39.52, H, 4.34, N, 3.55, Br, 40.45; found C, 39.68, H, 4.50, N, 3.45, Br, 40.20.

N-(*t-Butyloxycarbonyl*)-L-*3,5-dibromotyrosine t-butyl ester* [**23**].—To a solution of 3.00 g (7.59 mmol) of L-dibromotyrosine *t*-butyl ester in a mixture of 40 ml of $Et_2O$ and 40 ml of *t*-butyl alcohol, 1.80 ml (7.97 mmol) of di-*t*-butyl dicarbonate was added dropwise. The mixture was held at reflux for 5 h and then was concentrated *in vacuo* to give a hard glass, which crystallized upon scratching. The product was recrystallized from low-boiling petroleum ether to give 3.08 g (82%) of **23**: $[\alpha]^{22}$D +45.7° (*c*=0.161, MeOH); mp 114.5–115.5°; ir (Nujol) ν max 3440, 3260 (br), 1740, 1690, 1111, 1062, 1007 cm$^{-1}$; $^1$H nmr ($CDCl_3$) δ 1.43 [18H, 2 s, 2-C($CH_3$)$_3$], 2.94, 3.00 (2H, 2 overlapping dd, *J*=14.0 and 5.4 Hz, H-β), 4.36 (1H, br dt, *J*=7.5 and 5.6 Hz, H-α), 5.05 (1H, br d, *J*=7.5 Hz, NH), 5.84 (1H, s, OH), 7.26 (s, no integral, H-2, H-6); $^{13}$C nmr ($CDCl_3$) δ 28.0 [-C($CH_3$)$_3$], 28.3 [-C($CH_3$)$_3$], 36.8 (C-β), 54.7 (C-α), 79.9 [-*O*C($CH_3$)$_3$], 82.7 [-*O*C($CH_3$)$_3$], 109.5 (C-3, C-5), 131.0 (C-2, C-6), 132.9 (C-1), 148.3 (C-4), 154.9 (-NHCOO-), 170.3 (-COO-); *anal*., calcd for $C_{18}H_{25}Br_2NO_5$, C, 43.66, H, 5.09, Br, 32.27, N, 2.83; found C, 43.66, H, 5.06, Br, 32.23, N, 2.82.

[Methyl-$^{14}$*C*]-L-*3,5-dibromo*-O-*methyltyrosine* [**26**].—The break-seal ampoule that 100 μCi of [$^{14}$C]methyl iodide (specific activity 54 mCi/mmol) was supplied in was cooled in a dry ice/*i*-PrOH bath. An ice-cooled mixture of 13.84 mg (27.9 μmol) of **23**, 8.34 mg (60.3 μmol) of $K_2CO_3$, and 15.62 mg (59.1 μmol) of 18-crown-6 in 1 ml of dry $C_6H_6$ and a magnetic stirring bar were added to the ampoule, the ampoule was sealed, and the mixture was stirred at room temperature for 2 days. Then 200 μl of unlabeled $CH_3I$ was added, and the mixture was stirred an additional day. The reaction was poured into 1 ml of $H_2O$, the layers were separated, and the organic layer was stirred with 0.5 ml of 2 N NaOH for 1 h. The organic layer was washed with 0.5 ml of brine and concentrated *in vacuo*. The residue was taken up in 300 μl of $CH_2Cl_2$, 200 μl of trifluoroacetic acid was added, the vial was sealed, and the mixture was stirred overnight. Rtlc showed that the reaction was complete. The mixture was concentrated with a stream of $N_2$ to give 84 μCi (84%) of $^{14}$C-labeled **26**.

[Methyl-$^{13}$*C*]-L-*3,5-dibromo*-O-*methyltyrosine* [**26**].—To an ice-cold mixture of 0.801 g (1.62 mmol) of **23**, 0.247 g (1.79 mmol) of $K_2CO_3$, and 0.400 g (1.51 mmol) of 18-crown-6 in 25 ml of dry $C_6H_6$, was added 140 μl (2.23 mmol) of [$^{13}$C]methyl iodide. The mixture was stirred at room temperature for 6 h and poured into 20 ml of $H_2O$. The organic layer was washed with brine (2×15 ml), dried over $Na_2SO_4$, and concentrated. The product was taken up in 10 ml of $CH_2Cl_2$ and 10 ml of trifluoroacetic acid was added. The mixture was stirred overnight, concentrated, and taken up in $H_2O$. The pH was adjusted to 6.0 (pH meter) with $NH_4OH$ and the precipitate was dried in an Abderhalden pistol (110°) to give 0.560 g (98%) of **26**: $[\alpha]^{22}$D +0.94° (*c*=4.92, 1 N HCl); ir (Nujol) ν max 3200, 3050, 1660, 1200, 1187, 1150, 1131, 840, 803, 728 cm$^{-1}$; $^1$H nmr ($D_2O$, DCl) δ 3.04 (1H, dd, *J*=14.6 and 7.5 Hz), 3.14 (1H, dd, *J*=14.6 and 5.9 Hz), 3.80 (3H, d, $J_{(C\text{-}H)}$=147.1 Hz, $OCH_3$), 4.13 (1H, apparent t, *J*=7.1 and 6.3 Hz), 7.45 (2H, s); $^{13}$C nmr ($D_2O$, DCl) δ 60.8.

[Cyano-$^{13}$*C*]-p-*hydroxybenzonitrile* [**28**].—A mixture of 5.79 g (33.2 mmol) of *p*-bromophenol and 3.00 g (33.2 mmol) of cuprous [$^{13}$C]cyanide (19) in 50 ml of DMF was refluxed under $N_2$ for 24 h. To the cooled reaction mixture was added 50 ml of $H_2O$ followed by 1 N HCl until the mixture became turbid. The mixture was extracted with $Et_2O$ (75 ml×2) and the organic layer was washed with brine (75 ml) and dried over $Na_2SO_4$. The organic layer was concentrated *in vacuo* and the crude product was purified by Si gel cc [low-boiling petroleum ether-EtOAc (5:1)] to give 2.31 g (58%) of **28** as white crystals.

[Carboxy-$^{13}C$]-p-*hydroxybenzoic acid* [**29**].—A solution of 2.301 g (19.17 mmol) of **28** in 30 ml of concentrated HCl was refluxed for 4 h. Tlc [Si gel, $CHCl_3$-MeOH (4:1)] showed that no nitrile or amide was present in the reaction mixture. The solution was cooled in an ice bath and the crystals were collected and dried *in vacuo* to give 2.603 g (98%) of **29**.

*Methyl*[carboxy-$^{13}C$]-p-*methoxybenzoate* [**30**].—A solution of 2.600 g (18.70 mmol) of **29** in 50 ml of $Et_2O$ was treated with excess $CH_2N_2$ and left overnight. The reaction mixture was concentrated *in vacuo* to give 3.0 g (96%) of **30** as a white powder: $^1H$ nmr ($CDCl_3$) δ 3.84 (3H, s), 3.87 (3H, d, $J_{(C-H)}$=3.9 Hz), 6.90 (2H, d, $J$=8.7 Hz), 7.99 (2H, dd, $J$=8.8 Hz, $J_{(C-H)}$=3.9 Hz).

[Methylene-$^{13}C$]-p-*methoxybenzyl alcohol* [**31**].—A solution of 2.951 g (17.66 mmol) of **30** in 70 ml of dry $Et_2O$ was treated with 0.810 g (21.3 mmol) of $LiAlH_4$ and worked up in the usual manner to give 2.211 g (90%) of **31**: $^1H$ nmr ($CDCl_3$) δ 3.79 (3H, s), 4.58 (2H, trio, $J_{(C-H)}$=142.5 Hz, benzyl), 6.87 (2H, d, $J$=8.6 Hz), 7.27 (2H, dd, $J$=8.5 Hz, $J_{(C-H)}$=4.3 Hz, ArH-2,6). The $^{13}C/^{12}C$ ratio at the benzyl position was approximately 95:5 by nmr integration.

[Methylene-$^{13}C$]-p-*methoxybenzyl bromide* [**32**].—HBr was bubbled through a solution of 1.80 g (12.9 mmol) of **31** in 40 ml of dry $C_6H_6$ for 2 h. The mixture was stirred an additional 2 h under $N_2$ and concentrated *in vacuo* to give 1.76 g of **32** which was used immediately in the next step.

*Diethyl*[methylene-$^{13}C$]-*2-acetamido-2-*(p-*methoxybenzyl)malonate* [**33**].—To 20 ml of absolute EtOH was added 0.217 g (9.44 mmol) of Na followed by 2.04 g (9.39 mmol) of diethyl acetamidomalonate. The solution was cooled in an ice bath and 1.76 g of **32** were added. After being stirred overnight under $N_2$, 30 ml of $H_2O$ were added and the precipitate was collected and dried *in vacuo* to give 2.47 g (81%) of **33**: mp 94.5–95° [lit. (29) for diethyl 2-acetamido-2-[3′,5′-$^{13}C$]-*p*-methoxybenzylmalonate 96–97°]; ir (Nujol) ν max 3260, 1747, 1510, 1304, 1276, 1195, 846 $cm^{-1}$; $^1H$ nmr ($CDCl_3$) δ 1.26 (6H, t, $J$=7.1 Hz), 2.00 (3H, s), 3.56 (2H, d, $J_{(C-H)}$=134.2 Hz, benzyl), 3.74 (3H, s), 4.24 (4H, q, $J$=7.1 Hz), 6.50 (1H, s, NH), 6.76 (2H, d, $J$=8.6 Hz), 6.88, 6.90 (2H, dd, $J$=8.6 Hz, $J_{(C-H)}$=3.6 Hz, aryl); $^{13}C$ nmr ($CDCl_3$) δ 36.9.

D,L-[Methylene-$^{13}C$]*tyrosine*.—The diester **33** (2.47 g) was heated in 30 ml of refluxing 48% HBr for 4 h. The solution was concentrated *in vacuo* and dissolved in 15 ml of $H_2O$. The pH was adjusted to 5.5 (pH meter) with concentrated $NH_4OH$ and the precipitate was collected and dried *in vacuo* to afford 1.23 g (93%) of [*methylene*-$^{13}C$]-D,L-tyrosine: $^1H$ nmr ($D_2O$, DCl) δ 3.18 (1H, ddd, $J$=14.8 and 7.4 Hz, $J_{(C-H)}$=132.0 Hz, benzyl), 3.30 (1H, ddd, $J$=14.8 and 5.5 Hz, $J_{(C-H)}$=132.0 Hz, benzyl), 4.33–4.39 (1H, complex), 6.92 (2H, d, $J$=8.4 Hz), 7.22 (2H, dd, $J$=8.4 Hz, $J_{(C-H)}$=3.8 Hz, ArH-2, 6).

*Resolution of* D,L-[methylene-$^{13}C$]*tyrosine* [**29**].—A solution of 1.15 g (6.31 mmol) of D,L-[*methylene*-$^{13}C$]tyrosine in 6.0 ml of trifluoroacetic acid was cooled in an ice bath and 3.5 ml of $Et_2O$ was added, followed by 1.5 ml of trifluoroacetic anhydride. The mixture was stirred for 3 h and concentrated *in vacuo* and the residue was taken up in $Et_2O$ and filtered to remove the unreacted Tyr salt. The filtrate was concentrated *in vacuo* and the residue was recrystallized from $Et_2O$/toluene to give 1.42 g (82%) of *N*-trifluoroacetyl-D,L-[*methylene*-$^{13}C$]Tyr.

A 1.25-g portion of trifluoroacetyl-D,L-[*methylene*-$^{13}C$]Tyr was taken up in 50 ml of $H_2O$, the pH was adjusted to 7.8 with 2 N LiOH, and the mixture was stirred at 37°. A 250-ml aliquot of carboxypeptidase A, Type II-DFP (54 units/mg, 16.9 mg/ml) was concentrated to dryness with a stream of $N_2$ and combined with the solution. After 24 h the pH was adjusted to 5.5 (pH meter) with glacial HOAc and the mixture was cooled in an ice bath and filtered. The precipitate was dissolved in 1 N HCl, treated with Norite, and filtered through Celite, and the pH was adjusted to 5.5 with concentrated $NH_4OH$. The precipitate was collected and the Norite treatment was repeated to give 382 mg (88%) of L-[*methylene*-$^{13}C$]Tyr. A 5-mg portion of the product was suspended in 0.2 ml of MeOH and excess acetyl chloride was added. The stirred mixture was heated in a sealed vial at 110° for 1 h, and the solvent was then removed with a stream of $N_2$. To the vial 0.2 ml of trifluoroacetic acid and 50 ml of trifluoroacetic anhydride were added, and the solution was heated at 110° for 30 min. The solvent was removed with a stream of $N_2$ and the residue was dissolved in $CH_2Cl_2$. Chiral gc analysis showed that the product was greater than 99% of the L-isomer.

The combined mother liquors from the original precipitation of L-Tyr were adjusted to pH 3 with 2 N HCl and then concentrated *in vacuo*. The residue was crystallized from $Et_2O$/toluene to give 576 mg (96%) of *N*-trifluoroacetyl-D-Tyr. The trifluoroacetyl group was removed by heating in 5 ml of 3 N HCl at 100° for 2 h. The solution was decolorized as before, the pH was adjusted to 5.5 with $NH_4OH$, and the precipitate was collected and dried *in vacuo* to give 248 mg (62%) of D-[*methylene*-$^{13}C$]Tyr. The product was derivatized for chiral gc analysis as described above for the L-isomer, and the product was greater than 99% of the D-isomer.

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