Biosynthesis of Microcystin-LR. Origin of the Carbons in the Adda and Masp Units

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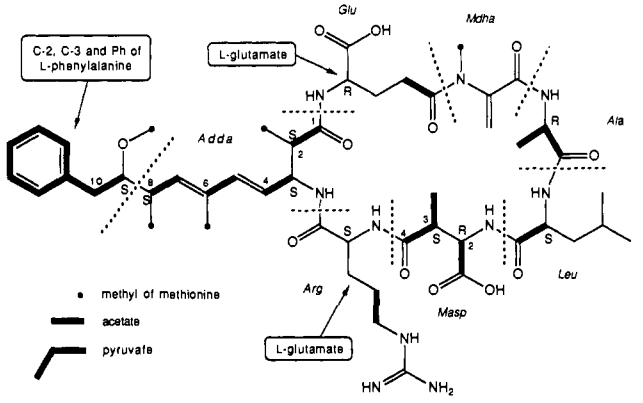
Cyclic heptapeptides known as microcystins[1] are responsible for the potent hepatotoxicity of certain blue-green algae (cyanobacteria).[2] Microcystin-LR [cyclo(D-Ala-L-Leu-D-β-Masp-L-Arg-Adda-D-γ-Glu-Mdha), **1**],[3] for example, is the major hepatotoxin associated with most strains of *Microcystis aeruginosa* Kützing found in the Northern Hemisphere. Recently microcystin-LR has been found to be a highly effective inhibitor of protein phosphatases 1 and 2A,[4] and this activity is believed to be closely associated with its hepatotoxicity.[5] We report here some initial findings on the biosynthesis of microcystin-LR in *M. aeruginosa* PCC-7820,[6] specifically on the origin of the carbons in the unusual (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid[7] (Adda) unit and the iso-linked (2*R*,3*S*)-3-methylaspartic acid (Masp) residue.



Origin of carbons in microcystin-LR (1)

The Me groups on C6 and C8 of Adda are methionine-derived[8] as shown by feeding experiments (see supplementary material) with L-[*methyl*-^{13}C]methionine. The Me group on C2 of Adda, however, appears to arise from two sources, one of which is methionine. This is indicated from the following two experiments: In one experiment where L-[*methyl*-^{13}C]methionine and unlabeled acetate were administered simultaneously to the alga, the C2 methyl carbon was enriched to the same extent as the C6 and C8 methyl carbons, suggesting that intact acetate is assimilated into C1 and C2 prior to methylation by methionine at C2. However, when L-[*methyl*-^{13}C]methionine was fed to the alga alone, incorporation of label into the C2 methyl carbon was quite small compared with the ^{13}C-enrichment of the C6 and C8 methyl carbons, suggesting that the Me on C2 was being derived from another precursor (possibly propionate[9]) in the absence of exogenous acetate.

Further feeding experiments established that the C3–C8 segment of Adda is acetate-derived, but more importantly that two pathways to the C1–C2 unit exist, one of which utilizes acetate. The ^{13}C NMR spectrum of toxin produced from feeding sodium [1,2-^{13}C]acetate (diluted 3-fold with unlabeled acetate) to the alga in one portion about 10 days after inoculation and harvesting the alga 2–4 days later showed that intact acetate was incorporated essentially equally into C1–C2 ($J_{1,2}$ = 49.9 Hz), C3–C4 ($J_{3,4}$ = 47.2 Hz), C5–C6 ($J_{5,6}$ = 54.1 Hz), and C7–C8 ($J_{7,8}$ = 44.4 Hz). However, when the alga was harvested 2 weeks after inoculation with acetate, incorporation of intact acetate into C1–C2 was low compared to uptake into C3–C8, suggesting that a different precursor (possibly propionate[9]) was being incorporated into C1–C2 as the concentration of exogenous acetate diminished. The remaining carbons in the Adda unit were shown to be phenylalanine-derived on the basis of a feeding experiment with [U-^{13}C]-L-phenylalanine. Although a feeding experiment with [1-^{13}C]phenylacetic acid[10] was unsuccessful, probably due to failure of this precursor to be absorbed into the alga, phenylacetyl-CoA has been concluded to be the most probable initiator for Adda biosynthesis.

[1,2-^{13}C]Acetate was assimilated into C4 and C5 of the γ-Glu ($^1J_{4,5}$ = 52.7 Hz) and Arg ($^1J_{4,5}$ = 36.1 Hz) units, but not into C3 and C4 of the β-Masp unit. Moreover, [1,2-^{13}C]-L-glutamic acid labeled C1 and C2 of the γ-Glu ($^1J_{1,2}$ = 54.5 Hz) and Arg ($^1J_{1,2}$ = 53.1 Hz) units, but not C1 and C2 of the β-Masp unit. These experiments indicated that Masp was not arising from rearrangement of glutamic acid.[11]

[1,2-^{13}C]Acetate was incorporated into C1 and C2 of Masp ($^1J_{1,2}$ = 55 Hz), however, and we conclude from this labeling pattern that Masp is synthesized as follows: Acetyl-CoA and pyruvic acid condense to 2-hydroxy-2-methylsuccinic acid, which is then converted to 2-hydroxy-3-methylsuccinic acid in a manner analogous to the conversion of (2*S*)-2-hydroxy-2-isopropylsuccinic acid to (2*R*,3*S*)-2-hydroxy-3-isopropylsuccinic acid in leucine

(1) (a) Konst, H.; McKercher, P. D.; Gorham, P. R.; Robertson, A.; Howell, J. *Can. J. Comp. Med. Vet. Sci.* **1965**, *29*, 221–228. (b) Carmichael, W. W.; Beasley, V.; Bunner, D. L.; Eloff, J. N.; Falconer, I.; Gorham, P.; Harada, K.; Krishnamurthy, T.; Min-juan, Y.; Moore, R. E.; Runnegar, M.; Skulberg, O. M.; Watanabe, M. *Toxicon* **1988**, *26*, 971.

(2) (a) Carmichael, W. W. In *Handbook of Natural Toxins*; Tu, A. T., Ed.; Marcel Dekker: New York, 1988; Vol. 3, pp 121–147. (b) Carmichael, W. W. In *Natural Toxins: Characterization, Pharmacology and Therapeutics*; Ownby, C. L., Odell, G. V., Eds.; Pergamon: London, 1988; pp 3–16.

(3) (a) Botes, D. P.; Tuinman, A. A.; Wessels, P. L.; Viljoen, C. C.; Kruger, H.; Williams, D. H.; Santikarn, S.; Smith, R. J.; Hammond, S. J. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2311. (b) Botes, D. P.; Wessels, P. L.; Kruger, H.; Runnegar, M. T. C.; Santikarn, S.; Smith, R. J.; Barna, J. C. J.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2747. (c) Rinehart, K. L.; Harada, K.; Namikoshi, M.; Chen, C.; Harvis, C. A.; Munro, M. H. G.; Blunt, J. W.; Mulligan, P. E.; Beasley, V. R.; Dahlem, A. M.; Carmichael, W. W. *J. Am. Chem. Soc.* **1988**, *110*, 8557.

(4) (a) MacKintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P.; Codd, G. A. *FEBS Lett.* **1990**, *264*, 187. (b) Hunkanen, R. E.; Zwiller, J.; Moore, R. E.; Daily, S. L.; Khatra, B. S.; Dukelow, M.; Boynton, A. L. *J. Biol. Chem.* **1990**, *265*, 19401.

(5) Yoshizawa, S.; Fujiki, H.; Matsushima, R.; Watanabe, M. F.; Harada, K.; Ichihara, A.; Carmichael, W. W. *J. Cancer Res. Clin. Oncol.* **1990**, *116*, 609.

(6) Codd, G. A.; Carmichael, W. W. *FEMS Microbiol. Lett.* **1982**, *13*, 409.

(7) Namikoshi, M.; Rinehart, K. L.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W. *Tetrahedron Lett.* **1989**, *30*, 4349.

(8) For actinobacteria, C_1 branches on polyketide carbon chains generally arise from C3 of propionate; in lankacidin-producing *Streptomyces* [Kakinuma, K.; Uzawa, J.; Uramoto, M. *Tetrahedron Lett.* **1982**, *23*, 5303], however, the *C*-methyl groups are methionine-derived. The C_1 branches of polyketides from fungi are usually methionine-derived, as are the non-acetate-derived C_1 branches of pseudomonic acid A from the Gram-negative bacterium *Pseudomonas fluorescens* [(a) Feline, T. C.; Jones, R. B.; Mellows, G.; Phillips, L. *J. Chem. Soc., Perkin Trans. 1* **1977**, 309. (b) Martin, F. M.; Simpson, T. J. *J. Chem. Soc., Perkin Trans. 1* **1989**, 207]. Interestingly the three branched *C*-methyl groups (C20, C21, and C22) of the fungal metabolite asteltoxin are methionine-derived, but its terminal methyl group (C1) can arise from either methionine or propionate (Steyn, P.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1984**, 977–979).

(9) Sodium [2,3-^{13}C]propionate and [2,3-^{13}C]succinic acid were not incorporated into **1**, at least under the conditions described in the supplementary material. In one feeding experiment where a toxic amount of sodium [1-^{13}C]propionate was fed to the alga, however, there appeared to be a significant incorporation of label into C1 of Adda.

(10) In the blue-green alga *Anacystis nidulans*, phenylacetate is biosynthesized from L-phenylalanine via phenylpyruvate (Löffelhardt, W. *Z. Naturforsch.* **1977**, *32C*, 345).

(11) In anaerobic bacteria, e.g., *Clostridium tetanomorphum*, (2*S*,3*S*)-3-methylaspartic acid is formed from L-glutamic acid (Hartrampf, G.; Buckel, W. *FEBS Lett.* **1984**, *171*, 73).

0002-7863/91/1513-5083$02.50/0

biosynthesis.[12] 2-Hydroxy-3-methylsuccinic acid is next oxidized to 2-oxo-3-methylsuccinic acid and finally transaminated to Masp. The incorporation of intact [U-^{13}C]pyruvate into C3, C4, and the Me on C3 of Masp ($^1J_{3,4}$ = 49.7 Hz and $^1J_{3,Me}$ = 34.1 Hz) supports the proposed biogenesis.[13]

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Supplementary Material Available: COSY ^{13}C NMR spectrum of **1** uniformly enriched with ^{13}C to 80% and ^{15}N to 90%, experimental details for feeding experiments, and ^{13}C NMR spectra of **1** labeled from precursors mentioned in this communication (15 pages). Ordering information is given on any current masthead page.

(12) (a) Calvo, J. M.; Stevens, C. M.; Kalyanpur, M. G.; Umbarger, H. E. *Biochemistry* **1964**, *3*, 2024. (b) Cole, F. E.; Kalyanpur, M. G.; Stevens, C. M. *Biochemistry* **1973**, *12*, 3346. (c) Sylvester, S. R.; Stevens, C. M. *Biochemistry* **1979**, *18*, 4529.

(13) [1,2-^{13}C]Acetate is also incorporated into C1 and C2 of the Leu unit of **1** ($^1J_{1,2}$ = 51.1 Hz). The specific incorporation is comparable with that of acetate into C1 and C2 of Masp. [U-^{13}C]Pyruvate is also incorporated intact into Ala.

Spiropentadiene

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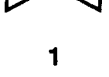
As the simplest small-ring, spiro-connected cycloalkene,[2] spiropentadiene (bowtiediene, **1**) is of considerable theoretical interest[3] since the two π systems are held in perpendicular planes by a common carbon and are predicted to experience significant spiroconjugation.[4] In view of the high energy content[5] of **1**, it is not surprising that very few attempts to synthesize this molecule have been reported.[6] We have demonstrated recently that the vacuum gas phase elimination of β-halocyclopropylsilanes by solid fluoride provides an attractive route to strained cycloalkenes.[6,7] We now report that this versatile technique can be applied to the synthesis of **1**.



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(1) American Chemical Society, Division of Organic Chemistry Fellow, sponsored by Rohm and Haas (1990-1991).

(2) For a review and discussion of spiroalkenes, see: Greenburg, A.; Liebman, J. F. *Strained Organic Molecules*; Academic: New York, 1978; p 104.

(3) For some recent references, see: (a) Kao, J.; Radom, L. *J. Am. Chem. Soc.* **1978**, *100*, 760. (b) Bischof, P.; Gleiter, R.; Haider, R. *J. Am. Chem. Soc.* **1978**, *100*, 1036. (c) Maksic, Z. B.; Kovacevic, K.; Mogus, A. *THEOCHEM* **1982**, *2*, 9. (d) Zil'berg, S. P.; Ioffe, A. I.; Nefedov, O. M. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1983**, 261. (e) Minkin, V. I.; Minyaev, R. M.; Orlova, G. M. *THEOCHEM* **1984**, *19*, 241. (f) Kanda, K.; Koremoto, T.; Imamura, A. *Tetrahedron* **1986**, *42*, 4169.

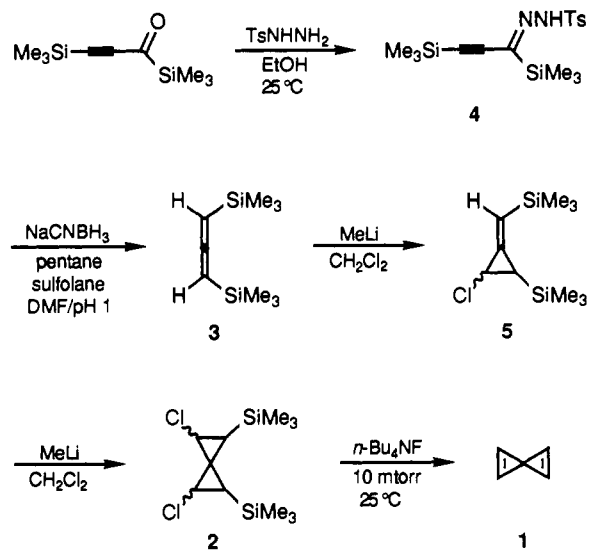
(4) (a) Simmons, H. E.; Fukunaga, T. *J. Am. Chem. Soc.* **1967**, *89*, 5208. (b) Hoffmann, R.; Imamura, A.; Zeiss, G. D. *J. Am. Chem. Soc.* **1967**, *89*, 5219.

(5) A range of values from 110 to 120 kcal/mol have been calculated.[3a,c,d] For a much higher estimate, see: Dewar, M. G. S. *The Molecular Orbital Theory of Organic Chemistry*; McGraw-Hill: New York, 1970; p 461.

(6) Billups, W. E.; Lin, L.-J. *Tetrahedron* **1986**, *42*, 1575.

(7) (a) Billups, W. E.; Haley, M. M. *Angew. Chem.* **1989**, *101*, 1735; *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 1711. (b) Billups, W. E.; Haley, M. M.; Arney, B. E., Jr.; Lee, G.-A.; Boese, R.; Bläser, D. *J. Org. Chem.*, in preparation. For other applications of the VGSR technique, see: (c) Denis, J.-M.; Niamayoua, R.; Vata, M.; Lablache-Combier, A. *Tetrahedron Lett.* **1980**, *21*, 515. (d) Billups, W. E.; Lin, L.-J.; Casserly, E. W. *J. Am. Chem. Soc.* **1984**, *106*, 3698. (e) Staley, S. W.; Norden, T. D. *J. Am. Chem. Soc.* **1984**, *106*, 3699. (f) Lacombe, S.; Gonbeau, D.; Caboich, J.-L.; Pellerin, B.; Denis, J.-M.; Pfister-Guillouzo, G. *J. Am. Chem. Soc.* **1988**, *110*, 6964.

Scheme I



Spiropentane **2** was envisioned as the immediate precursor to **1**. The synthesis of **2** is presented in Scheme I. The key intermediate required for this synthesis is the sterically hindered allene **3**.[8] Fortunately, this compound could be prepared readily via the procedure described by Danheiser and his co-workers to synthesize (trimethylsilyl)allene.[9] Thus pure **3** was isolated in 57% yield by treatment of the tosylhydrazone of bis(trimethylsilyl)propynone[10] (**4**)[11] with sodium cyanoborohydride in a two-phase solvent system at pH 1. Treatment of the allene **3** with chlorocarbene, generated from methyllithium and dichloromethane,[12] yielded compound **5** in 14% yield.[11] Conversion of **5** to the desired spiropentane **2** was effected by subjecting **5** to the same reaction conditions, yielding **2** as a viscous oil in 6% yield after column chromatography.

Introduction of **2** into a "fluoride column"[13] using the VGSR apparatus described previously[6] yielded a volatile hydrocarbon which could be condensed into a liquid nitrogen trap as a white solid. Examination of the hydrocarbon by ^{1}H NMR spectroscopy at −105 °C in tetrahydrofuran-d_8 revealed a singlet at δ 7.62 (cyclopropenyl protons) along with several unidentified signals. The singlet at δ 7.62 was observed to disappear after approximately 20 min at −105 °C in THF-d_8; rapid decomposition occurred upon warming to −90 °C. We were unable to record the ^{13}C NMR spectrum of **1**.

On warming, **1** polymerizes to a sparingly soluble (THF), light green film. The propensity of spiropentadiene to polymerize is not unexpected since spiropentene is reported to polymerize in the condensed phase at −78 °C.[14]

(8) Allene **3** has been prepared previously by reacting the anion of bis(trimethylsilyl)propyne with sodium sulfate decahydrate with subsequent separation of the resulting allene–propyne mixture by HPLC: Peterson, P. E.; Jensen, B. L. *Tetrahedron Lett.* **1984**, *25*, 5711.

(9) Danheiser, R. L.; Carini, D. J.; Fink, D. M.; Basak, A. *Tetrahedron* **1983**, *39*, 935.

(10) Kruithof, K. J. H.; Schmitz, R. F.; Klumpp, G. W. *Tetrahedron* **1983**, *39*, 3073.

(11) Proton (300 MHz) and carbon-13 (75.5 MHz) NMR spectral data of new compounds are as follows. **2**: ^{1}H NMR 3.68-3.11 (m, 2 H), 0.99-0.82 (m, 2 H), 0.21-0.13 (3 s, 18 H); ^{13}C NMR 40.5, 39.8, 39.3, 32.4, 31.4, 17.9, 17.6, 14.1, −0.1, −0.2, −1.7. **4**: ^{1}H NMR 8.70 (s, 1 H), 7.82 (d, 2 H, J = 8.2), 7.30 (d, 2 H, J = 8.2), 2.43 (s, 3 H), 0.23 (s, 9 H), 0.15 (s, 9 H); ^{13}C NMR 146.3, 144.0, 135.6, 129.4, 127.9, 113.6, 94.8, 21.6, −0.4, −2.7. **5**: ^{1}H NMR 6.18 (d, 1 H, J = 3.1), 3.71 (d, 1 H, J = 9.3), 1.34 (dd, 1 H, J = 9.3, 3,1), 0.18 (s, 9 H), 0.14 (s, 9 H); ^{13}C NMR 141.6, 117.5, 32.5, 17.0, −0.4, −0.9. **6**: ^{1}H NMR 5.80 (dd, 2 H, J = 5.3, 3.0), 5.73 (dd, 2 H, J = 5.3, 3.0), 2.75 (br s, 2 H), 2.70 (br s, 2 H), 1.77 (dt, 2 H, J = 6.6, 1.8), 1.66 (d, 2 H, J = 6.6), 1.39 (d, 2 H, J = 4.0), 1.36 (d, 2 H, J = 4.0); ^{13}C NMR 134.5, 132.1, 63.5, 44.2, 43.4, 29.7, 24.7, 20.5.

(12) Closs, G. L.; Closs, L. E. *J. Am. Chem. Soc.* **1960**, *82*, 5723.

(13) A "fluoride column" was prepared by depositing 2.0 g of tetra-*n*-butylammonium fluoride on 20 g of glass helices as described in ref 6.

(14) Bloch, R.; Denis, J.-M. *Angew. Chem.* **1980**, *92*, 969; *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 928.

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