Tetrahedron 67 (2011) 877–880

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Complete ^{13}C-labeling pattern of yessotoxin a marine ladder-frame polyether

Masatoshi Yamazaki, Kazuo Tachibana, Masayuki Satake *

Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 18 November 2010
Received in revised form 4 December 2010
Accepted 7 December 2010
Available online 10 December 2010

Keywords:
Biosynthesis
Ladder-frame polyether
^{13}C-Labeling
Carbon deletion

ABSTRACT

Biosynthesis of a marine ladder-frame polyether yessotoxin (YTX) produced by the dinofalgellate *Protoceratium reticulatum* was investigated. The ^{13}C-labeling experiments indicated that the carbons in YTX were derived from acetates, a methyl of methionine and glycolate, and six-membered ring tetrads (rings A–D and H–K) were constructed from repetition of C3 units (m–m–c), which consisted of a methyl of acetate and acetate.

1. Introduction

Marine dinoflagellates produce a variety of intriguing secondary metabolites.[1] Those compounds are fascinating to a diverse group of scientific fields not only because of their unique and complicated structures but also their potent and specific biological activities. Numerous efforts have been directed toward the synthesis of these marine natural products using various strategies,[2] but compared with such chemical studies, their biological aspects, such as mode of action and biosynthetic studies are not as well developed. Only a few biosynthetic studies of dinoflagellate metabolites have been reported about okadaic acid and its derivatives,[3] goniodomin,[4] amphidinolides,[5] 13-desmethylspirolide C,[6] and amphidinols[7] whose structural features are classified into a polyether carboxylic acid, a macrolide, and a linear polyene-polyol. Interestingly, the ^{13}C incorporation patterns are unique and quite different from those of polyketides obtained from fungi and bacteria.[8] The carbon backbone of these dinoflagellate metabolites is constructed from acetate (m–c) units though often the starter group is glycolate or glycine. More unusual is the observation that the common sequence of acetate units is interrupted by acetate methyl-derived carbons arising between the intact units. A truncated methyl–methyl (m–m) coupling in the polyketide chain is a striking feature of dinoflagellate polyketides and frequently occurs near branching carbons, such as pendant methyls and exomethylenes on the carbon chain in okadaic acids and amphidinols. The m–m coupling has been proposed to be formed by decarboxylation of an acetate carbonyl by a flavin-mediated Favorskii rearrangement[3d,7a] or oxidative decarboxylation.[9]

Ladder-frame polyethers, such as brevetoxins, ciguatoxins, and maitotoxin are also a group of the representative secondary metabolites produced by dinoflagellates.[1] The characteristic structural feature of ladder-frame polyethers is the arrangement of contiguous *trans*-fused ether rings of various sizes from five- to nine-membered rings. The extremely limited yield of these dinoflagellate metabolites has hampered studies of their biosynthesis, and consequently biosynthetic studies of ladder-frame polyethers have been limited to brevetoxin-B.[10] The ^{13}C-acetate incorporation pattern of brevetoxin-B was complicated and six m–m couplings and two m–m–m moieties were observed.

Yessotoxin (YTX, Fig. 1a) is a disulfated ladder-frame polyether,[11] and was first isolated from scallops in Japan nearly twenty years ago, but later the dinoflagellate *Protoceratium reticulatum* was confirmed as a YTX producer.[12] Although its biosynthesis was unknown, YTX was considered a suitable candidate for biosynthetic studies of dinoflagellate ladder-frame polyethers because it has two simple six-membered ring tetrad sequences (rings A–D and H–K) and two medium size ether rings (rings E and G) in the molecule. Furthermore, the six-membered ring tetrad occurs in many of ladder-frame polyethers, and it was reasoned that incorporation of ^{13}C-acetate units into the six-membered ring tetrad might illustrate a basic and common carbon chain construction mechanism of biosynthesis for the ladder-frame polyethers. To this end we have investigated the biosynthesis of YTX and report the complete ^{13}C-labeling pattern in YTX following incorporation of various labeled precursors.

* Corresponding author. Tel.: +81 3 5841 4357; fax: +81 3 5841 8380; e-mail address: msatake@chem.s.u-tokyo.ac.jp (M. Satake).

0040-4020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2010.12.015

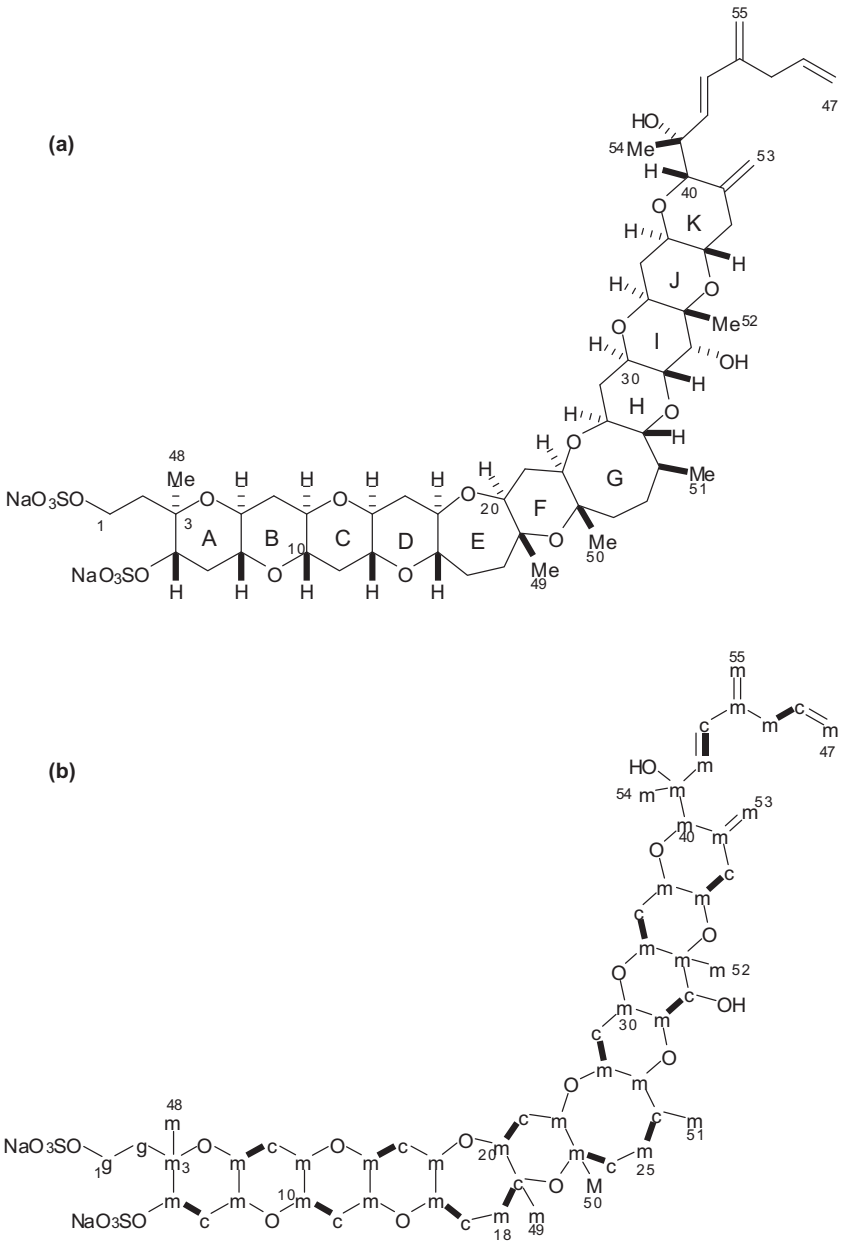

Fig. 1. (a) Structure of yessotoxin (YTX). (b) ^{13}C-labeling patterns of YTX (m: acetate methyl; c: acetate carbonyl; M: methionine methyl; g: glycolate). Bold lines indicate incorporation of intact acetate units.

2. Results and discussion

The dinoflagellate *P. reticulatum* was collected in Mutsu Bay, Aomori Prefecture, Japan, and cultured in 2 L nutrient-enriched seawater medium. Feeding experiments were carried out with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$]acetates, [^{13}C-*methyl*]methionine, and [1-^{13}C]glycolate. For each labeling experiment, the dinoflagellate was supplemented with 25 mg/L of ^{13}C-labeled precursors at the time of inoculation, and *P. reticulatum* was cultured for 40 days. Harvested cells were extracted with MeOH. After evaporation of MeOH, the extracts were partitioned EtOAc/H_2O and then BuOH/H_2O. The BuOH fraction containing YTX was purified using an alumina column and then a series of C_{18} columns.[12] From 10 L cultures of the dinoflagellate cells, nearly 1 mg of ^{13}C-labeled YTX was obtained.

The ^{13}C NMR spectra of YTX from cultures supplemented with ^{13}C-acetates showed that incorporation of ^{13}C-acetate in each experiment was uniform and sufficiently large. Isotope enrichment was estimated to be 4–8% by calculation of MALDI MS peaks. Comparison of ^{13}C peak intensity of non-labeled and ^{13}C-acetate labeled YTX at the each position indicated that 52 of 55 carbons in YTX were labeled by the acetate precursors (Fig. 1b). Incorporation of [2-^{13}C]acetate resulted in labeling of 37 carbons. All the bridgehead carbons except for C19 were labeled with [2-^{13}C]acetate and C1 branching groups, five out of six pendant methyls and two exomethylenes were also labeled with the [2-^{13}C]acetate. Incorporation of [1-^{13}C]acetate resulted in labeling of 15 carbons. All methylene carbons on the six-membered ether rings were derived from the carboxyl carbon of an intact acetate unit, but in contrast only two oxycarbons (C19, C32) were labeled by [1-^{13}C]acetate. The incorporation experiment of [1,2-$^{13}C_2$]acetate indicated that 15 intact acetate units, which were readily assigned from $^1J_{C,C}$ values were incorporated into the skeletal structure (Table 1) and carbon chain construction started from the C1 and C2 portion. Three

Table 1
^{13}C NMR assignment of YTX in CD_3OD ($^{13}CD_3OD$ at 49.8 ppm)

No.	δ_C	Intensity ratio[a] (labeled/unlabeled)		$^1J_{C,C}$ [Hz]	Origin	No.	δ_C	Intensity ratio[a] (labeled/unlabeled)		$^1J_{C,C}$ [Hz]	Origin
		[1-^{13}C]-Acetate	[2-^{13}C]-Acetate					[1-^{13}C]-Acetate	[2-^{13}C]-Acetate		
1	65.8	0.8	0.3	s	g	28	84.9	1.1	3.3	35	m
2	41.1	1.7	0.9	s	g	29	40.8	4.1	0.6	35	c
3	77.4	0.3	2.0	s	m	30	74.1	1.3	3.1	s	m
4	79.6	1.3	2.5	35	m	31	80.4	0.5	3.0	43	m
5[b]	33.7	2.3	2.0	34	c	32	74.6	5.1	0.8	43	c
6	79.3	1.9	2.2	s	m	33	77.6	0.5	2.8	s	m
7	71.4	0.6	2.9	35	m	34	74.1	1.3	3.1	39	m
8	37.3	4.3	0.7	35	c	35	32.5	3.4	0.9	40	c
9	79.0	0.4	3.2	s	m	36	73.9	0.9	3.0	s	m
10[c]	79.1	1.9	2.2	37	m	37	73.8	0.6	3.7	34	m
11	37.0	4.4	0.7	37	c	38	39.8	2.4	0.8	35	c
12	78.4	1.2	3.0	s	m	39	144.0	1.2	2.2	s	m
13	78.9	0.5	3.0	36	m	40	85.9	0.2	2.9	s	m
14	38.8	3.0	1.7	36	c	41	79.1	0.4	3.2	s	m
15	82.0	0.9	3.1	s	m	42	137.5	0.7	2.5	73	m
16	83.0	0.4	3.0	39	m	43	131.4	3.1	0.6	74	c
17	30.1	3.9	1	40	c	44	146.2	0.1	2.3	s	m
18	41.9	1.0	3.1	41	m	45	38.6	0.6	3.1	40	m
19[c]	79.2	1.9	2.2	41	c	46	138.4	2.3	0.3	41	c
20	83.2	0.6	4.0	35	m	47	117.3	0.9	3.3	s	m
21	33.9	3.9	1.2	34	c	48	16.9	0.8	2.8	s	m
22	88.2	1.2	3.4	s	m	49	24.7	1	3.4	s	m
23	77.8	0.7	3.1	40	m	50	21.5	0.5	0.9	s	M
24	47.9	3.4	0.7	41	c	51	23.1	0.7	3.3	s	m
25[b]	33.7	2.3	2.0	37	m	52	16.1	0.2	3.0	s	m
26	41.7	3.4	1.1	37	c	53	116.4	1.6	3.2	s	m
27	90.2	0.8	3.5	s	m	54	27.0	0.7	3.3	s	m
						55	117.3	0.9	3.3	s	m

[a] Intensity of each peak in the labeled YTX divided by that of the corresponding signal in the unlabeled YTX, normalized to give a ratio of 1 for an unenriched peak (C49 for [1-^{13}C]acetate labeling and C17 for [2-^{13}C]acetate labeling).
[b] These signals were overlapped with each other.
[c] These signals were overlapped with each other.

carbons at C1, C2, and Me50 were not labeled with acetates. Instead, the bridgehead methyl group Me50 was heavily labeled following incorporation of [^{13}C-*methyl*]methionine. Finally C2 was labeled with [1-^{13}C]glycolate indicating that glycolate is a starter unit for the biosynthetic process (Fig. 2). Together, these data account for the origin of all the carbons in YTX.

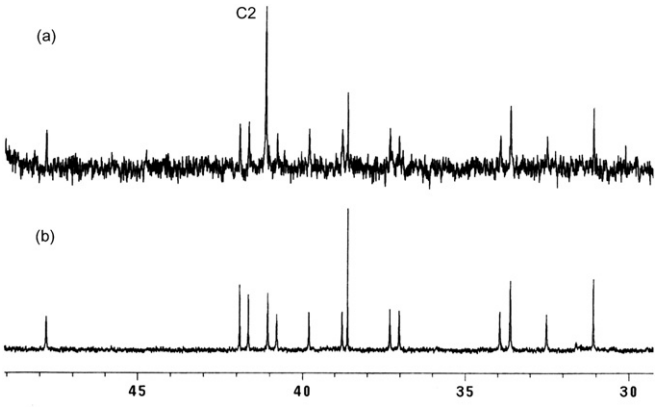

Fig. 2. Partial ^{13}C NMR spectra of YTX. (a) YTX incorporated from [1-^{13}C]glycolate, (b) non-labeled YTX.

Although 15 direct acetate units were embedded in the carbon chain, the common uninterrupted acetate sequence (m–c–m–c…) following Claisen condensation of intact acetate units was observed only in a short stretch of the carbon backbone (C16–C21) of YTX. Instead, m–m coupling resulting from carbon deletion of an acetate unit was observed at 14 sites in the carbon backbone. Interestingly, the two six-membered tetrads, rings A–D and H–K were constructed by the repeated condensation of a C3 unit (m–m–c) formed from a methyl group of a deleted acetate unit followed by an intact unit. This observation suggests that the C3 unit is a basic building block of the ladder-frame polyethers to build the common six-membered ring and that insertion of an intact acetate unit can change the ring size from a six-membered ring to a seven- or eight-membered ether ring (e.g., C21–C20–C19–C18 and C26–C25). Thus, the carbon deletion process occurs regularly after acetate condensation to elongate a carbon chain in the biosynthesis of the ladder-frame polyethers. In contrast the basic building block of the non-ladder-frame dinoflagellate polyketides, such as okadaic acid and the amphidinols is acetate and the m–m coupling is mainly seen around pendant methyls and exomethylenes. This work reveals not only the unique carbon chain construction of marine ladder-frame polyethers but also the difference of the carbon chain assembly process between ladder-frame and non-ladder-frame marine polyketides.[13]

3. Experimental section

3.1. Chemicals and instruments

All labeled compounds were purchased from Shoko Co. Ltd. Other chemicals were obtained from Wako Pure Chemicals and used without further purification. ^{13}C NMR spectra were measured in CD_3OD and pyridine-d_5 with a JEOL ECX400 spectrometer (^{13}C at 100 MHz) and a Varian INOVA 600 spectrometer (^{13}C at 150 MHz). MALDI MS spectra were obtained on a Shimadzu-Kratos AXIMA-CFR mass spectrometer.

3.2. Cultivation, labeling and isolation

The dinoflagellate *P. reticulatum* was collected at Aomori Prefecture, Japan. The dinoflagellate was cultured at 17 °C for 40 days in a seawater medium enriched with GSe nutrients. Feeding experiments were carried out with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$] acetates (sodium salts), [^{13}C-*methyl*]methionine, and [1-^{13}C]glycolate (sodium salt). For each labeling experiment, the dinoflagellate was supplemented with 25 mg/L of ^{13}C-labeled precursor at the time of inoculation. The harvested cells by centrifugation were extracted with MeOH. After evaporating MeOH, the extract was partitioned between H_2O and ethyl acetate. The aqueous layer was extracted with butanol. The butanol fraction was evaporated, dissolved in $CHCl_3$–MeOH (1:1), and passed through an alumina column (Wako Pure Chemicals, Japan). The column was washed with $CHCl_3$–MeOH (1:1) and MeOH, and finally YTX was eluted with 1% NH_4OH–MeOH (1:1). The eluent was passed through a Sep-Pak ODS (Waters) with 80% MeOH. Final purification of YTX was done on a Develosil ODS UG-5 column (Nomura Chemicals, Japan, 4.6×250 mm) with MeOH–MeCN–H_2O (2:1:2).

Acknowledgements

We are grateful to Prof. Wright J. L. C., University of North Carolina, Wilmington for helpful discussion. This work was supported by the Global COE Program for Chemistry Innovation, the University of Tokyo and KAKENHI.

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.12.015.

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