HOKKAIDO UNIVERSITY

Title	Biosynthetic assembly of cytochalasin backbone
Author(s)	Fujii, Ryuya; Minami, Atsushi; Gomi, Katsuya; Oikawa, Hideaki
Citation	Tetrahedron Letters, 54(23), 2999-3002 https://doi.org/10.1016/j.tetlet.2013.03.120
Issue Date	2013-06-05
Doc URL	http://hdl.handle.net/2115/52893
Type	article (author version)
File Information	13TL-cytochalasin-final.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

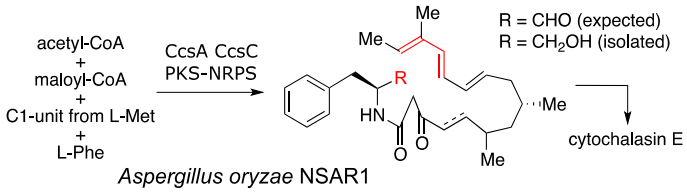
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Biosynthetic Assembly of Cytochalasin Backbone

Leave this area blank for abstract info.

Ryuya Fujii, Atsushi Minami, Katsuya Gomi and Hideaki Oikawa



Biosynthetic Assembly of Cytochalasin Backbone

Ryuya Fujii[a] , Atsushi Minami[a] , Katsuya Gomi[b] and Hideaki Oikawa[a, *]

[a]*Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[b]*Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Cytochalasin
Biosynthesis
Polyketide synthase
Non-ribosomal peptide synthethase
Fungal metabolite

ABSTRACT

Cytochalasins are an important class of fungal natural products in view of structural diversity and biological activities. Although their biosynthetic studies have been examined extensively, the detailed molecular assembly mechanism remained to be solved. We have succeeded to heterologously express the cytochalasin polyketide synthase-non-ribosomal peptide synthethase (PKS-NRPS) hybrid gene *ccsA* and the trans-acting enoyl-CoA reductase gene *ccsC* in *A. oryzae*. The resultant transformant produced a novel metabolite possessing the cytochalasin backbone. This established that CcsA is capable of constructing the octaketide connected with phenylalanine in collaboration with CcsC, and that CcsA R domain catalyzes reductive cleavage of the thio-tethered PKS-NRPS product.

2009 Elsevier Ltd. All rights reserved.

1. Introduction

A family of cytochalasan members represented by cytochalasin A[1] (Fig. 1) is well known mycotoxins widely distributed in various fungi.[2] Its potent inhibition of the actin polymerization affects a wide range of cellular events and thus cytochala synthetically sins are important biochemical tools for studying fundamental cellular processes.[2] Molecular diversity of cytochalasan family members such as cytochalasin, chaetoglobosin, aspochalasin is derived from three parts; 1) a pyrrolidinone consisting of distinct amino acid; 2) a cyclohexane with functionalities derived from epoxide; 3) a macrocycle including 11- and 13-membered carbocycles, a lactone and a carbonate with various oxidative decorations (Fig. 1).[2-4]

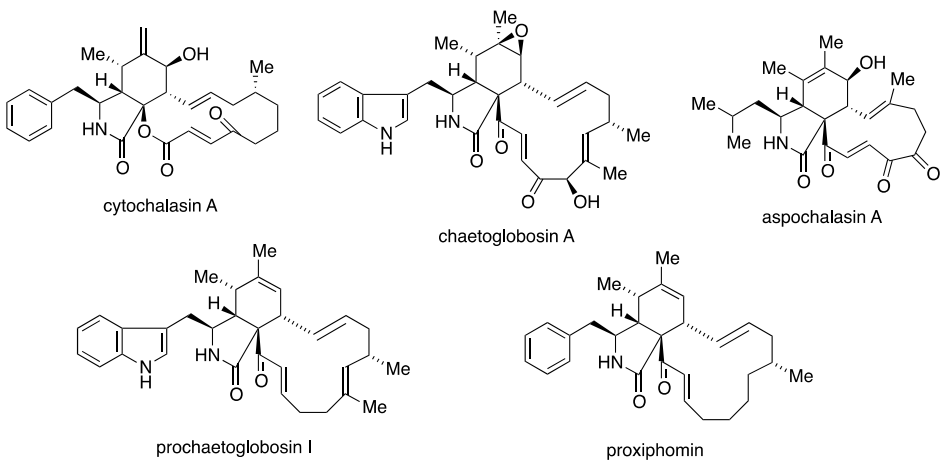
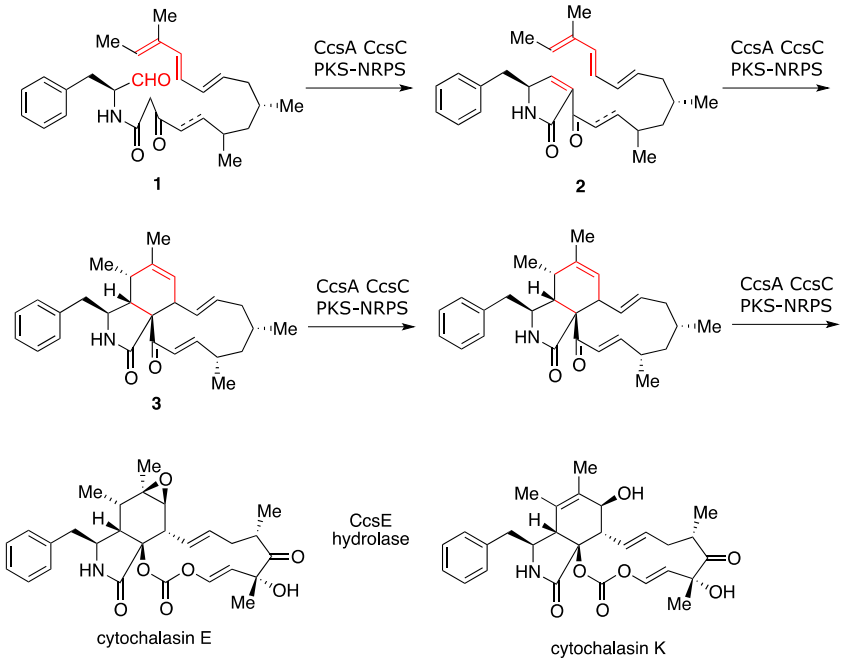


Figure 1. Structures of representative cytochalasan family metabolites from various fungi.

* Corresponding author. Tel.: +81-11-706-2622; fax: ++81-11-706-3448; e-mail: hoik@sci.hokudai.ac.jp



Scheme 1. Proposed late step biosynthetic pathway of cytochalasins E and K in *Aspergillus clavatus* NRRL1.

The biosynthetic origins of cytochalasan molecular skeleton were established by a number of feeding experiments with isotope-labeled precursors such as acetate and amino acids.[3,4] Although involvement of the intramolecular Diels-Alder reaction (IMDA) was proposed for construction of the characteristic perhydro-isoindolone with macrocyclic system in the cytochalasin biosynthesis, it was difficult to define the reaction sequence in the late stage modifications.[2-4] In the biosynthetic study of the structurally related cheatoglobosin, we succeeded to determine the origin of oxygen atoms[5] and to obtain a series of less oxidized analogs[6] including prochaetoglobosin I by treatment of the producer strain with the specific P450 inhibitors (Fig.1). These results enabled us to propose IMDA of the pyrrolinone with linear polyketide chain to give prochaetoglobosin I.[5] In 2007, using an RNA silencing method, Schumann and Hertweck identified the first cytochalasan biosynthetic gene cluster[7] consisting of a polyketide synthase-non-ribosomal peptide synthethase (PKS-NRPS) hybrid and oxidative modification enzymes which are expected in our proposal.

Recently, Tang and co-workers successfully identified the biosynthetic gene cluster of cytochalasin E[8] in *Aspergillus clavatus* NRRL1.[9] This cluster consisted of eight genes for the backbone construction (*ccsA*: PKS-NRPS, *ccsC*: trans-acting enoyl-CoA reductase (trans-ER)), modification enzymes (*ccsBDG*: oxidative modifications, *ccsE*: hydolase), and transcription factor (*ccsR*) whose functions are reasonably assigned except hypothetical protein (*ccsF*) (Schemes 1, 2). These proposals are essentially same as the proposed biosynthetic pathway of chaetoglobosins.[5,7] Our continuous interest on a Diels-Alderase prompted us to examine the molecular assembly mechanism of cytochalasin. Herein, we describe novel metabolites from the transformants by introducing two genes (*ccsA* and *ccsC*) in a heterologous host *Aspergillus oryzae* and discuss the construction mechanism of the cytochalasin core scaffold.

2. Results and Discussion

To date, only three PKS-NRPS hybrids such as TenS (tenellin),[10] CpaS (cyclopiazonic acid),[11] ApdA (aspyridone)[12] were successfully expressed in heterologous hosts and studied in detail. TenS and ApdA require trans-ER (TenC, ApdC) for complementation of the inactive modular ER. To examine the function of the cytochalasin PKS-NRPS CcsA, we expressed *ccsA* and the trans acting ER gene *ccsC*. For this purpose, we used the auxotrophic mutant *A. oryzae* NSAR-1[13] aiming to the reconstitution of cytochalasin machinery as in the case of other fungal natural products.[14] After preparing the expression plasmids pTAex3[15]-*ccsA*, and pUSA[16]-*ccsC*, the resultant plasmids were transformed into the *A. oryzae* NSAR-1 in a stepwise manner. The ccsA/ccsC transformant was cultured in CD medium supplemented with maltose to induce target gene expression.[14a] HPLC analysis of the partially purified fraction from the mycelial extracts showed a new peak that were not found in a control culture of the wild-type strain (Fig. 3). To determine their structures, the mycelial acetone extract of the *ccsA/ccsC* double transformant obtained from large-scale incubation using solid medium was partitioned with hexane-acetonitrile. The resultant acetonitrile extract was purified by silica gel column chromatography and by reverse-phase HPLC to give the major product **4** (3.0 mg/kg medium).

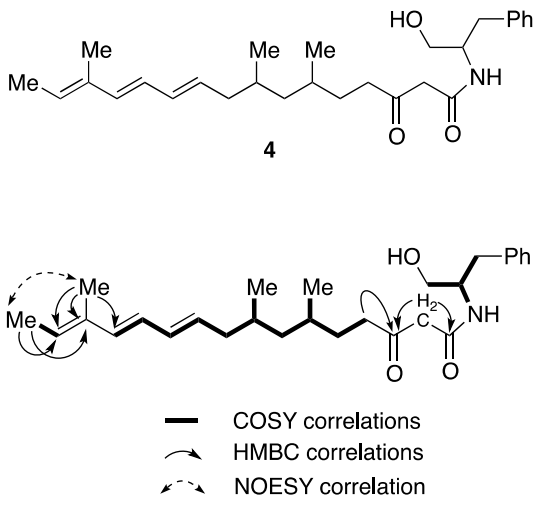


Figure 2. Structure and NMR data of metabolite 1 from ccsA/ccsC double transformants.

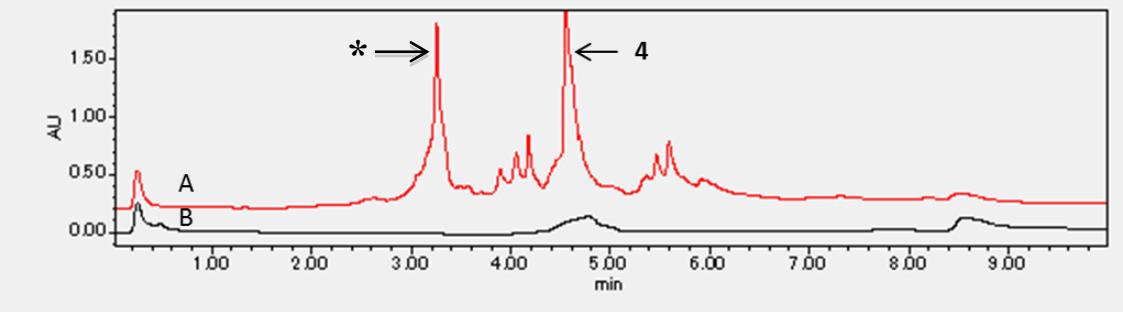


Figure 3. HPLC analysis of mycelial extracts from *A. oryzae* NSAR1. A) Partially purified extracts from a transformant carrying *ccsA* and *ccsC* genes: B) extracts from wild type strain. * is an unidentified peak that is neither **1** nor **2** (ESI-MS analysis). Crude extracts were analyzed by a Water UPLC system including a ACQUITY UPLC BEH C18 (1.7 µm, φ2.1 mm x 150 mm). A gradient of water and acetonitrile solvent system was used. 10 µL of the extract solution was injected for each round 15 minutes HPLC program (0 min, 20% CH_3CN; 5 min, 90% CH_3CN, 10 min, 90% CH_3CN, 12 min, 20% CH_3CN, 15 min, 20% CH_3CN)

Table 1. NMR data of the compound 4 (500 MHz, C_6D_6)

Carbon No.	δ_C	δ_H	(mult, *J* in Hz)
1	167.2		
2	50.2	2.78	(d, 16.7)
		2.85	(d, 16.7)
3	207.5		
4	42.4	1.93	(t, 7.5)
5	32.3	1.42	(m)
		1.21	(m)
6	31.0	1.68	(m)
7	45.5	1.08	(m)
		1.17	(m)
8	32.1	1.29	(m)
9	42.7	2.07	(dd, 13.8, 6.3)
		1.96	(dd, 13.8, 7.3)
10	133.2	5.68	(dt, 14.6, 7.3)
11	134.3	6.22	(dd, 14.6, 7.0)
12	127.9	6.31	(m)
13	137.5	6.32	(m)
14	136.3		
15	127.9	5.52	(q, 7.1)
16	15.1	1.58	(d, 7.1)
17	20.7	0.69	(d, 6.5)
18	20.4	0.85	(d, 6.5)
19	13.3	1.70	(s)
1’	65.5	3.47	(dd, 10.9, 3.8)
		3.37	(dd, 10.9, 5.3)
2’	54.8	4.23	(m)
3’	38.4	2.70	(d, 7.5)
4’	139.6		
5’	130.8	7.26	(m)
6’	129.9	7.27	(m)
7’	129.7	7.20	(m)
8’	129.9	7.27	(m)
9’	130.8	7.26	(m)
NH		6.90	(d, 7.0)

HR-EIMS of the isolated **4** indicated a molecular formula of $C_{28}H_{41}NO_3$ (unsaturation: 9). ^{1}H- and ^{13}C-NMR spectral data (Table 1) indicated the presence of phenyl group (δ_H 7.20-7.27, m, 5H), two ketone (δ_C 207.5) and amide (δ_C 167.18) carbonyl groups, suggesting that its structure is closely related with that of cytochalasin. Extensive NMR data analysis, including COSY, HSQC, and HMBC, enabled us to determine the structure of **4** as shown in Fig. 2. Observed NOEs between the terminal (C16) and the branched (C19) methyl groups established the stereochemistry of trisustituted olefin as *E*. The structure thus determined is nearly identical to a proposed octaketide intermediate **1** consisting of a conjugated triene, three branched methyl group and a β-ketoamide connected with phenylalanine. For formation of putative pyrrolinone precursor **2**, Hertweck and co-workers proposed two routes; route A proceeds via Claisen condensation, reduction and dehydration; route B via reduction and Knoevenagel condensation (Scheme 2).[2b] The structure of **4** suggested that the CcsA reductase (R) domain reductively liberated the putative thioester intermediate appended to the CcsA thiolation (T) domain to give **1**. Isolation of proxiphomin[17] and procheatoglobosin I[6] (Fig. 1), non oxidatively modified putative precursors in the corresponding cytochalasan producing fungi, provides the circumstantial evidence that the first free linear intermediate has an enone moiety.

Isolation of acyclic alcohol **4** clearly indicated that the PKS-NRPS CcsA is responsible for the cytochalasin backbone assembly in collaboration with the trans-ER CcsC, and also provided the first direct evidence for involvement of the linear intermediate before the putative Diels-Alder reaction and the Bayer-Villiger oxidation(s) (Schemes 1 and 2). Our preliminary examination failed to detect putative Diels-Alder adduct **3** in the extracts of the *ccsA/ccsC* transformant. This suggests that the *ccsF* whose function is not assigned catalyzes both Knoevenagel condensation and [4+2] cycloaddition. The reduced product **4** can be converted into putative intermediate analogs dihydro-**1** and pyrrolinone dihydro-**2** via oxidation of primary alcohol and the subsequent Knoevenagel condensation. Therefore, isolation of **4** opens a door to investigate the intriguing IMDA in vitro using the candidate enzyme including CcsF.

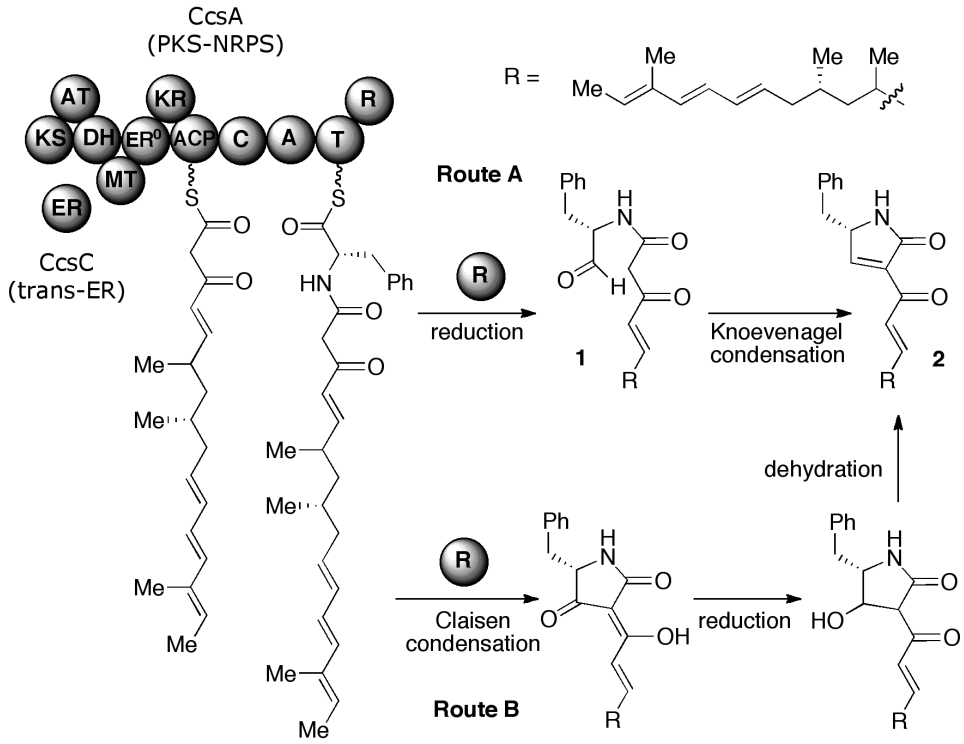
3. Conclusion

In summary, we have succeeded to heterologously express the cytochalasin PKS-NRPS *ccsA* and the trans aciting ER *ccsC* in *A. oryzae*. The resultant transformant produced metabolite **4** possessing the cytochalasin backbone. This established that CcsA is capable for constructing the octaketide connected with phenylalanine in collaboration with CcsC, and that the CcsA R domain catalyzes reductive cleavage of thio-tethered PKS-NRPS product. The product obtained in this study may enable a rapid access to the IMDA substrate. Currently, we are working on in vitro study of the putative cytochalasin Diels-Alderase most likely CcsF.

4. Acknowledgments

This work was supported by the Grants-in-Aid for Scientific Research 22108002 to H. Oikawa from Japan Society for the

Promotion of Science (JSPS). A fellowship to R.F. from the JSPS is gratefully acknowledged.



Scheme 2. Proposed biosynthetic assembly of cytochalasin backbone in *Aspergillus clavatus* NRRL1.

Supplementary Material

Supplementary material (detailed experimental procedures and NMR data including various 2D NMR spectra) associted with this article can be found, in the online version, at xxxxxxxxxxxxx.

References and notes

1. (a) Rothweiler, W.; Tamm, C. *Experientia* **1966,** *22*, 750; (b) Aldridge, D. C.; Armstrong, J. J.; Speak, e. N.; Turner, W. B. *Chem. Commun.* **1967**, 26.
2. (a) Tanenbaum, S. W. Ed. *Cytochalasins, Biochemicaland Cell Biological Aspects*; Tanenbaum, S. W., Ed.; North-Holland Publishing Co.: Amsterdam, 1978; (b) Scherlach, K.; Boettger, D.; Remme, N.; Hertweck, C. *Nat. Prod. Rep.* **2010**, *27*, 869.
3. (a) Binder, M.; Tamm, C. *Angew. Chem., Int. Ed.* **1973**, *12*, 370; (b) Tamm, C. *Front. Biol*. **1978**, *46*, 15.
4. (a) Probst, A.; Tamm, C. *Helv. Chim. Acta* **1981**, *64*, 2065; (b) Sekita, S.; Yoshihira, K.; Natori, S. *Chem. Pharm. Bull.* **1983**, *31*, 490.
5. Oikawa, H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2955.
6. (a) Oikawa, H.; Murakami, Y.; Ichihara, A. *Tetrahedron Lett.* **1991**, *32*, 1349; (b) Oikawa,' H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2949.
7. Schumann, J.; Hertweck, C. *J. Am. Chem. Soc.* **2007**, *129*, 9564.
8. Buchi, G.; Kitaura, Y.; Yuan, S. S.; Wright, H. E.; Clardy, J.; Demain, A. L.; Ginsukon, T.; Hunt, N.; Wogan, G. N. *J Am Chem Soc* **1973**, *95*, 5423.
9. Qiao, K. J.; Chooi, Y. H.; Tang, Y. *Metab. Eng.* **2011**, *13*, 723.
10. Halo, L. M.; Marshall, J. W.; Yakasai, A. A.; Song, Z.; Butts, C. P.; Crump, M. P.; Heneghan, M.; Bailey, A. M.; Simpson, T. J.; Lazarus, C. M.; Cox, R. J. *ChemBioChem* **2008**, *9*, 585.
11. (a) Seshime, Y.; Juvvadi, P. R.; Tokuoka, M.; Koyama, Y.; Kitamoto, K.; Ebizuka, Y.; Fujii, I. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3288; (b) X. Y.; Walsh, C. T. *Biochemistry* **2009**, *48*, 8746.
12. Xu, W.; Cai, X. L.; Jung, M. E.; Tang, Y. *J. Am. Chem. Soc*. **2010**, *132*, 13604.
13. Jin, F. H.; Maruyama, J.; Juvvadi, P. R.; Akioka, M.; Kitamoto, K. *FEMS Microbiol. Lett*. **2004**, *239*, 79.
14. (a) Fujii, R.; Minami, A.; Tsukagoshi, T.; Sato, N.; Sahara, T.; Ohgiya, S.; Gomi, K.; Oikawa, H. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1813; (b) Matsuda, Y.; Awakawa, T.; Itoh, T.; Wakimoto, T.; Kushiro, T.; Fujii, I.; Ebizuka, Y.; Abe, I. *ChemBioChem* **2012**, *13*, 1738; (c) Tagami, K.; Liu, C.; Minami, A.; Noike, M.; Isaka, T.; Fueki, S.; Shichijo, Y.; Toshima, H.; Gomi, T.; Dairi, T.; Oikawa, H. *J. Am. Chem. Soc.* **2013**, *135*, 1260; (d) Chiba, R.; Minami, A.; Gomi, K.; Oikawa, H. *Org. Lett.* **2013**, *15*, 594.
15. Fujii, T.; Yamaoka, H.; Gomi, K.; Kitamoto, K.; Kumagai, C. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1869.
16. Yamada, O.; Na Nan, S.; Akao, T.; Tominaga, M.; Watanabe, H.; Satoh, T.; Enei, H.; Akita, O. *J. Biosci. Bioeng.* **2003**, *95*, 82.
17. Binder, M.; Tamm, C. *Helv. Chim. Acta* **1973**, *56* 2387.