Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 4273–4276

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

KMI-008, a Novel β-Secretase Inhibitor Containing a Hydroxymethylcarbonyl Isostere as a Transition-State Mimic: Design and Synthesis of Substrate-Based Octapeptides

Daisuke Shuto,[a] Soko Kasai,[a] Tooru Kimura,[a] Ping Liu,[a] Koushi Hidaka,[a] Takashi Hamada,[a] Saeko Shibakawa,[a] Yoshio Hayashi,[a] Chinatsu Hattori,[b] Beata Szabo,[b] Shoichi Ishiura[b] and Yoshiaki Kiso[a,*]

[a]*Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan*

[b]*Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Megro-ku, Tokyo 153-8902, Japan*

Received 19 August 2003; accepted 29 September 2003

Abstract—A novel class of substrate-based β-secretase (BACE1) inhibitors containing a hydroxymethylcarbonyl (HMC) isostere was designed and synthesized. Phenylnorstatine [(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; Pns] was an effective transition-state mimic at the P_1 position. Structure–activity relationships (SARs) of the P_3–P_3' positions of BACE1 inhibitors were studied.

Introduction

The accumulation of amyloid β peptide (Aβ) in the brain is a major factor in the pathogenesis of Alzheimer's disease (AD).[1] Aβ is formed by an initial cleavage of the β-amyloid precursor protein (APP) by β-secretase to form a membrane-bound C-terminal fragment, which is then proteolyzed further by γ-secretase to form Aβ (1–40) or Aβ (1–42) (Fig. 1).[2] Although the detailed characteristics and cleavage mechanism of γ-secretase have not been elucidated, β-secretase has been identified as a novel membrane-bound aspartic protease, BACE1 (memapsin 2, Asp2)[3–5] and the crystal structure of its catalytic domain was also determined.[6] BACE1 is an excellent target for developing drugs, since it occupies the initial step in the pathological cascade of AD. Several groups reported BACE1 inhibitors based on the transition-state analogues, such as hydroxyethylene[7] and statine[8,9] at the scissile peptide bond, which are known to be effective in studies of inhibitors of aspartic proteases. Herein, we report novel substrate-based octapeptide inhibitors of BACE1 containing a hydroxymethylcarbonyl isostere (HMC) as a transition-state mimic.

*Corresponding author. Tel.: +81-75-595-4635; fax: +81-75-591-9900; e-mail: kiso@mb.kyoto-phu.ac.jp

0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.09.053

Design and Synthesis

Based on the common enzymatic mechanism of aspartic proteases, substrate transition-state mimics have been proposed and are currently widely used for the design of highly potent aspartic protease inhibitors. HMC isostere, which is one of the typical substrate transition-state mimics, is normally inserted into the P1 position of the substrate with high affinity and selectivity towards the target aspartic protease, resulting in the conversion of substrates to inhibitors. In our previous study, potent inhibitors with α-hydroxy-β-amino acids that have the HMC structures have been developed towards several human disease-related aspartic proteases such as renin,[10] HIV protease[11,12] and plasmepsin II.[13] Along the same lines, we focused on the substrate sequence deduced from Swedish mutant APP. It was reported that Swedish mutant APP, which has the mutation at the P_2–P_1 positions from Lys-Met to Asn-Leu, results in about a 60-fold increase of the k_{cat}/K_M of β-secretase cleavage.[4] Our initial design was based on the sequence of Swedish mutant APP (P_4–P_4': EVNL*DAEF).

At first, we designed and synthesized an octapeptide **1** containing a α-hydroxy-β-amino acid, norstatine [(2*R*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid: Nst] at P_1 position (Fig. 1).[14] However, the BACE1

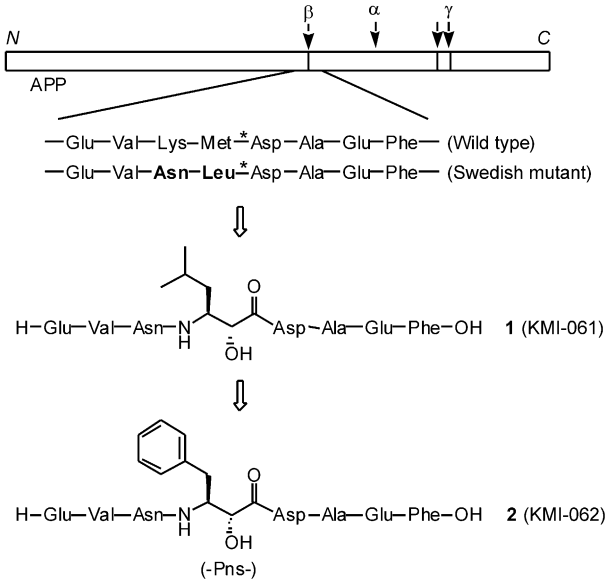


Figure 1. Design of substrate-based BACE1 inhibitors.

inhibitory activity of compound **1** was less than 20% at 2 μM (Table 1). Therefore, we tried to change the P_1 core of peptide **1** into phenylnorstatine [(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid: Pns][11] (Fig. 1). Since Pns, mimicked phenylalanine, had a larger side chain than Nst, a leucine mimetic, we expected that the contribution of the hydrophobic interaction at the S_1 subsite might increase. Compound **2** exhibited the BACE1 inhibitory activity (24% inhibition at 2 μM), thus we chose Pns as a transition-state mimic at the P_1 position. Furthermore, we designed several substrate-based octapeptides to study the SAR of the P_3–P_3' positions.

These compounds were synthesized by the Fmoc based solid phase method. For example, the synthesis of **2** (KMI-062) is shown in Scheme 1. We used Wang resin as a solid support and 1-hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIPCDI) as coupling reagents. Fmoc-Pns-OH was condensed without any protection of the hydroxyl group as described previously.[11] Deprotection and cleavage of peptide resin was done by treatment with trifluoroacetic acid (TFA) containing *m*-cresol and thioanisole. The obtained crude peptide was purified using RP-HPLC and characterized by MALDI-TOF MS. The synthetic peptides were adopted to enzyme assay using a recombinant human BACE1 and a fluorescence-quenching substrate.[15]

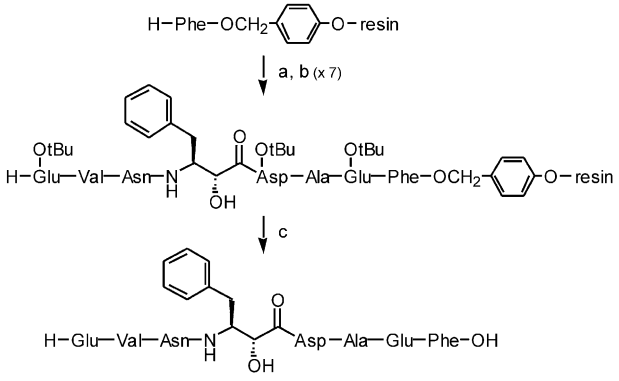
Results and Discussion

Compound **1** (KMI-061), which was an octapeptide containing HMC isostere as a transition-state analogue and amino acid sequence of Swedish mutant substrate (P_4–P_4'), had no inhibitory activity. However a substitution of Nst at the P_1-position of **1** into Pns gave a weak inhibitor **2** (KMI-062). We selected **2** as the lead compound and initially studied SAR for P_2 and P_3 positions. Inhibitory activities against BACE1 when the P_2 position changed to several other amino acids (compounds **3**–**8**) are shown in Table 1. In the case of hydrophilic amino acids (Asn, Asp, Glu, and Gln) in the P_2 position, the inhibitory activities of these inhibitors (compounds **3**–**5**) were weak (25–36%). The inhibitory activity of **8**, which contains a native P_2 amino acid (Lys) of APP, was also

Table 1. BACE1 inhibitory activities of octapeptide-type BACE1 inhibitors

Compd (KMI no.)	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	BACE1 inhibition (%)[a]
Substrate (Swedish)	H- Glu	-Val	-Asn	-Leu	-Asp	-Ala	-Glu	-Phe -OH	
1 (−061)				Nst					<20
2 (−062)				Pns					24
3 (−389)			Asp	Pns					25
4 (−391)			Gln	Pns					36
5 (−390)			Glu	Pns					30
6 (−392)			Met	Pns					42
7 (−008)			Leu	Pns					>90[b]
8 (−089)			Lys	Pns					<20
9 (−394)		Leu	Leu	Pns					<20
10 (−395)		Ile	Leu	Pns					26
11 (−396)		Phe	Leu	Pns					<20
12 (−393)		Met	Leu	Pns					<20
13 (−005)			Leu	Pns	Ala				53
14 (−085)			Leu	Pns	Asn				54
15 (−068)			Leu	Pns	Glu				64
16 (−086)			Leu	Pns	Gln				41
17 (−016)			Leu	Pns	Dmt				34
18 (−004)			Leu	Apns					66
19 (−001)			Leu	Apns	Ala				<20
20 (−109)			Leu	Apns	Asn				22
21 (−108)			Leu	Apns	Glu				47
22 (−110)			Leu	Apns	Gln				33
23 (−015)			Leu	Apns	Dmt				34
24 (−055)			Leu	Pns	Ala	Val			30
25 (−054)			Leu	Pns	Ala	Leu			41
26 (−053)			Leu	Pns	Ala	Phe			60
27 (−066)			Leu	Pns	Ala	Glu			78
28 (−106)			Leu	Pns			Gln		56
29 (−105)			Leu	Pns			Val		57
30 (−073)			Leu	Pns			Leu		57
31 (−072)			Leu	Pns			Phe		67

[a]At 2 μM.
[b]IC_{50} = 413 nM.



Scheme 1. Synthetic route to **2** (KMI-062): (a) Fmoc-AA-OH, DIPCDI, HOBt (2.5 equiv each)/DMF; (b) 20% piperidine/DMF; (c) TFA/*m*-cresol/thioanisole/H_2O (37:1:1:1).

weak. However, the compound **7** (KMI-008) containing a hydrophobic amino acid (Leu) in the P_2 position showed significant inhibitory activity (>90%). These results suggested that the hydrophobic interaction at the S_2 site of BACE1 was more effective than the hydrophilic one, in spite of the hydrophilic property of the S_2 site. Since **7** (KMI-008) showed good activity, Leu was employed as the P_2 moiety for the following experiments.

The S_3 site of BACE1 consists of some hydrophobic amino acids. In order to increase the hydrophobic interaction at the P_3 position, we replaced the original Val into some hydrophobic amino acids (Leu, Ile, Phe and Met). The inhibitory activities of **9**–**12** were extremely weak compared with **7**. These results showed that Val, which was the native amino acid of APP, fit better than other bulky amino acids and that the S_3 site had a small capacity.

Next, we studied the preference of the P_1' position which is important for specific recognition of the aspartic protease inhibitor. The P_1' position was changed by several amino acids (Ala, Asn, Glu and Gln). The preference of the P_1' position is clearly Asp (compound **7**) and **13**–**16** had moderate activities. It is assumed that Asp formed the ionic interaction or hydrogen bond at the S_1' site.

Previously, we reported that the inhibitors consisting of allophenylnorstatine [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid: Apns], a diastereomer of Pns, in the P_1 position and 5,5-dimethylthiazolidine-4-carboxylic acid (Dmt) in the P_1' position highly inhibited HIV-1 protease.[12] Therefore, we designed compounds **17**–**23**. Introduction of Dmt at the P_1' gave weak BACE1 inhibitors (**17** and **23**). The stereochemistry of the transition-state mimetic hydroxyl group is important for the inhibitory activity as described.[11] To examine a stereochemical preference of the hydroxyl group of HMC, **18**–**23** were prepared. The conversion of Pns of **7** to Apns yielded moderate active inhibitor **18** as compared with **7** (66% and >90%, respectively). In the case of **19**–**22**, the potency was decreased by the conversion. These results showed that the (2*R*)-hydroxyl group of HMC was better to obtain efficient inhibitory activity against BACE1, and Dmt was not preferred as the P1′ amino acid.

SAR for the P_2' and P_3' positions was also carried out. Although several hydrophilic and hydrophobic amino acids were introduced to the P_3' position, the inhibitory activities of these compounds (**28**–**31**) were slightly decreased and had almost same values (56–67%).

Similarly, substitutions at the P_2' position gave moderate active inhibitors (**24**–**27**). The preference at these positions was not strict and both inhibitors containing hydrophilic or hydrophobic moiety were active. These results showed that the P_2'–P_3' moieties of the inhibitors did not provide tight binding to BACE1.

From the above SAR study, compound **7** (KMI-008, H-Glu-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH) showed the highest activity (IC_{50} = 413 nM) among the synthesized octapeptides. The characteristic structures of KMI-008 are a central core of Pns at P_1 containing HMC and Asp at P_1' as compared to other published BACE1 inhibitors. For example, OM99-2[6] reported by Hong et al. has a leucine mimic transition-state analogue at the P_1 position and does not have Asp at the P_1' position. Hom et al.[9] reported inhibitors based on statine derivatives, which mimicked Leu-Gly or Phe-Gly in the P_1–P_1' positions. As illustrated in Figure 2, KMI-008 could provide good interaction between the BACE1 active site prepared from 1FKN (pdb data).[6] It will be a great advantage for the future design to obtain powerful inhibitors, because Asp at the P_1' position can form a tight interaction with the S_1' site. However, since long-chain peptide inhibitor KMI-008 may be metabolically unstable, it is necessary to minimize the number of natural peptide bonds and reduce the molecular weight.

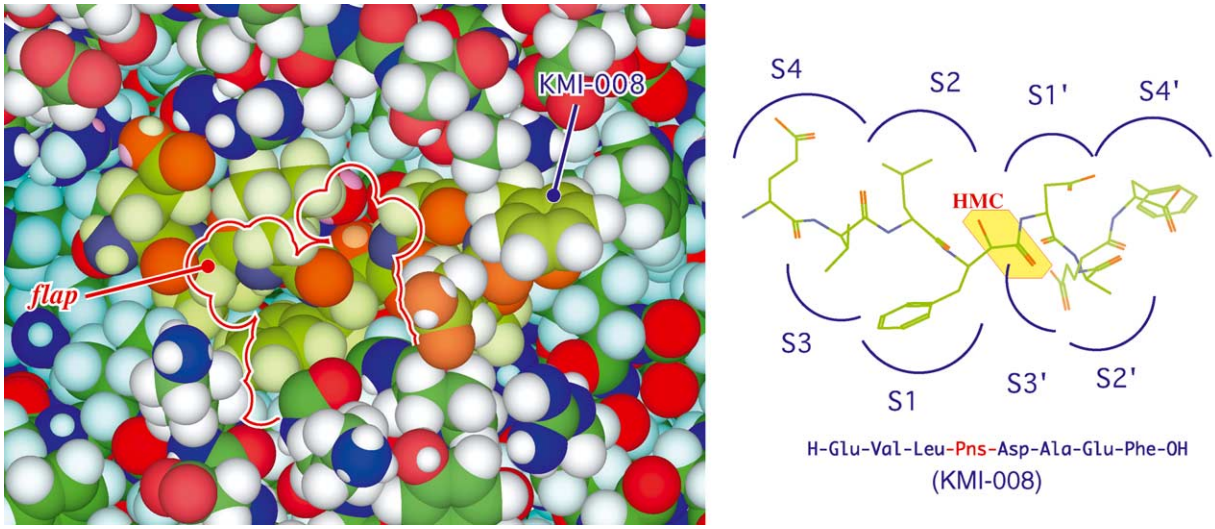


Figure 2. Modeled structure of KMI-008 bound to BACE1. The inhibitor molecule is shown in light green and the red line indicates the flap region of BACE1 (Tyr^{71}-Gly^{74}) placed over the inhibitor. The model was constructed using the crystal structure of BACE1 bound to OM99-2.[6]

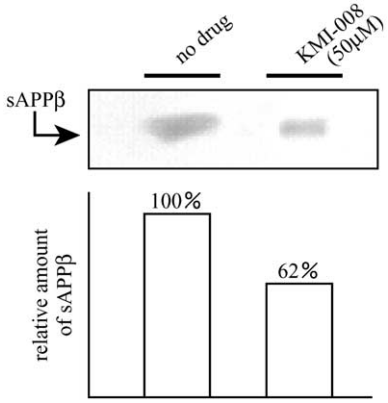


Figure 3. The effect of KMI-008 on β-secretase activity.

As a preliminary experiment, we studied the effect of KMI-008 on β-secretase activity in COS-7 cells transfected with APPwt and BACE1. The secretion of sAPPβ, a soluble form of APP generated by the action of β-secretase, from transfected cells was detected by anti-sAPPβ antibody.[16] KMI-008 reduced the secretion of sAPPβ at 50 μM concentration (Fig. 3).[17] This result showed the possibility that inhibitors containing HMC isostere could be used as drugs to reduce Aβ formation in vivo.

Conclusion

In conclusion, a novel class of BACE1 inhibitors were designed and synthesized using a 'hydroxymethylcarbonyl (HMC) isostere' as a transition-state mimic. KMI-008 showed remarkable BACE1 inhibitory activity and is a good lead compound in further design of BACE1 inhibitors.

Acknowledgements

This research was supported in part by the Frontier Research Program of the Ministry of Education, Science and Culture of Japan, and grants from the Ministry of Education, Science and Culture of Japan.

References and Notes

1. Selkoe, D. J. *Nature* **1999**, *399*, A23.
2. Sinha, S.; Lieberburg, I. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11049.
3. Vassar, R.; Bennet, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. *Science* **1999**, *286*, 735.
4. Lin, X.; Koelsch, G.; Wu, S.; Downs, D.; Dashti, A.; Tang, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1456.
5. Yan, R.; Bienkowski, M. J.; Shuck, M. E.; Miao, H.; Tory, M. C.; Pauley, A. M.; Brashier, J. R.; Stratman, N. C.; Mathews, W. R.; Buhl, A. E.; Carter, D. B.; Tomasselli, A. G.; Parodi, L. A.; Heinrikson, R. L.; Gurney, M. E. *Nature* **1999**, *402*, 533.
6. Hong, L.; Koelsch, G.; Lin, X.; Wu, S.; Terzyan, S.; Ghosh, A. K.; Zhang, X. C.; Tang, J. *Science* **2000**, *290*, 150.
7. Ghosh, A. K.; Bilcer, G.; Harwood, C.; Kawahama, R.; Shin, D.; Hussain, K. A.; Hong, L.; Loy, J. A.; Nguyen, C.; Koelsch, G.; Ermolieff, J.; Tang, J. *J. Med. Chem.* **2001**, *44*, 2865.
8. Tung, J. S.; Davis, D. L.; Anderson, J. P.; Walker, D. E.; Mamo, S.; Jewett, N.; Hom, R. K.; Sinha, S.; Thorsett, E. D.; John, V. *J. Med. Chem.* **2002**, *45*, 259.
9. Hom, R. K.; Fang, L. Y.; Mamo, S.; Tung, J. S.; Guinn, A. C.; Walker, E. D.; Davis, D. L.; Gailunas, A. F.; Thorsett, E. D.; Sinha, S.; Knops, J. E.; Jewett, N. E.; Anderson, J. P.; John, V. *J. Med. Chem.* **2003**, 1799.
10. Iizuka, K.; Kamijo, T.; Harada, H.; Akahane, K.; Kubota, T.; Umeyama, H.; Ishida, T.; Kiso, Y. *J. Med. Chem.* **1990**, *33*, 2707.
11. (a) Mimoto, T.; Imai, J.; Tanaka, S.; Hattori, N.; Takahashi, O.; Kisanuki, S.; Nagano, Y.; Shintani, M.; Hayashi, H.; Sakikawa, H.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1991**, *39*, 2465. (b) Mimoto, T.; Imai, J.; Tanaka, S.; Hattori, N.; Kisanuki, S.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1991**, *39*, 3088.
12. Mimoto, T.; Imai, J.; Kisanuki, S.; Enomoto, H.; Hattori, N.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1992**, *40*, 2251.
13. (a) Nezami, A.; Luque, I.; Kimura, T.; Kiso, Y.; Freire, E. *Biochemistry* **2002**, *41*, 2273. (b) Nezami, A.; Kimura, T.; Hidaka, K.; Kiso, A.; Liu, J.; Kiso, Y.; Goldberg, D. E.; Freire, E. *Biochemistry* **2003**, *42*, 8459.
14. Rich, D. H.; Moon, B. J.; Boparai, A. S. *J. Org. Chem.* **1980**, *45*, 2288.
15. Recombinant human BACE1 was purchased from R & D Systems, Minneapolis, MN, USA. Source: A cDNA sequence encoding the ectodomain (amino acid residues 1–460) of the recombinant human β-secretase[3,5] was expressed as a secreted protein with a COOH-terminal His tag in a murine myeloma cell line, NS0. Molecular Mass: The purified secreted rhBACE1 exists as two forms, pro and mature, with N-terminal sequence of TQHGIRLPLR and ETDEEPEEPG, respectively. By SDS-PAGE, the apparent molecular masses are approximately 75 kDa for the proform and 72 kDa for the mature form. Fluorescence-quenching substrate[18] (7-methoxycoumarin-4-yl)acetyl-Ser-Glu-Val-Asn-Leu*Asp-Ala-Glu-Phe-Arg-Lys(2,4-dinitrophenyl)-Arg-Arg-NH_2 for β-secretase was purchased from Peptide Institute Inc. Osaka, Japan. BACE1 inhibitory activity of the test compounds was determined based on the decrease% of the cleaved substrate by the enzyme. The assay was done in 2 μM inhibitor, 7 nM BACE1, 25 μM substrate, 50 mM MES, 50 mM CH_3COOH, NaOH (pH 5.0), 7% DMSO. After incubation for 60 min at 37 °C, the reaction was stopped by addition of trichloroacetic acid. The N-terminal cleavage fragment [(7-methoxycoumarin-4-yl)acetyl-Ser-Glu-Val-Asn-Leu-OH] was analyzed by RP-HPLC with the detection of fluorescence intensity (*Ex*, 328 nm; *Em*, 393 nm).
16. Hattori, C.; Asai, M.; Oma, Y.; Kino, Y.; Sasagawa, N.; Saido, T. C.; Maruyama, K.; Ishiura, S. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1228.
17. COS-7 cells were grown in DMEM containing 10% FBS and co-transfected with wild-type APP and full-length BACE1. As a control, pcDNA3HA empty vector was used. 48 h post-transfection, conditioned medium was replaced by new serum-free medium in the presence or absence of KMI-008, and incubated for 6 h. The culture media was concentrated by the TCA method and dissolved in SDS sample buffer. Samples were run on 7.5% SDS-PAGE and western blotted. The blots were probed with anti-sAPPβ antibody. The amount of the secreted proteins was normalized by the cell number.
18. Koike, H.; Seki, H.; Kouchi, Z.; Ito, M.; Kinouchi, T.; Tomioka, S.; Sorimachi, H.; Saido, T. C.; Murayama, K.; Suzuki, K.; Ishiura, S. *J. Biochem.* **1999**, *126*, 235.