Medicinal Chemistry

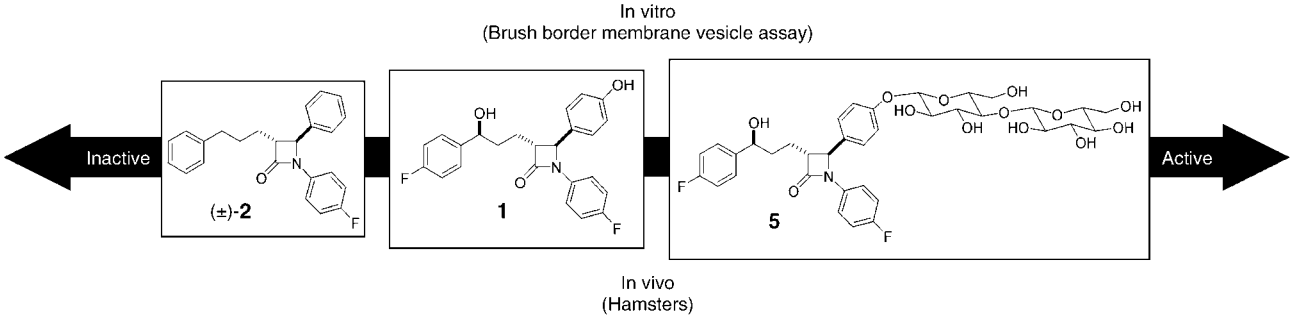
# An In Vitro Assay for Evaluation of Small-Molecule Inhibitors of Cholesterol Absorption**

*Lisbet Kværnø, Tobias Ritter, Moritz Werder, Helmut Hauser, and Erick M. Carreira**

Cardiovascular disease is the leading cause of death in the Western industrialized world and claims more lives than the next four causes combined.[1] A major risk factor for this disease is elevated cholesterol levels.[2,3] The current predominant therapy prescribes the use of statins, which lead to inhibition of cholesterol biosynthesis in the liver.[3,4] However, half of all patients undergoing current lipid-lowering treatments fail to reach their cholesterol goals,[3,5] and thus a clear need for the development of new cholesterol-lowering agents remains. Ezetimibe (**1**, Scheme 1) is a recent exciting example of a new class of drugs operating by a different mechanism involving inhibition of intestinal cholesterol absorption. Ezetimibe (**1**) was developed solely by using animal studies.[6] The rapid identification and optimization of new inhibitors would be greatly facilitated with an in vitro assay. In this communication, we introduce the use of intestinal brush border membrane vesicles[7] for the convenient in vitro testing of small molecules for inhibition of cholesterol absorption. We demonstrate the validity of the assay by comparison of the observed in vitro efficacies with published in vivo data (Scheme 1). Moreover, the assay allows us to identify new nonhydrolyzable glycosides as potent cholesterol absorption inhibitors and an oxazolidinone as an effective replacement of the β-lactam scaffold of ezetimibe (**1**).

The actual identity of the intestinal cholesterol transport proteins is still actively debated, with evidence suggesting the involvement of the scavenger receptors SR-BI[7e,f,8] and CD36,[7f,9] the Niemann–Pick C1-like 1 protein,[10] and an Annexin 2/Caveolin 1 complex.[11] Regardless of which proteins are involved, prior work has demonstrated that cholesterol absorption takes place in brush border membrane vesicles made from human or animal small intestines.[7] We thus set out to investigate whether brush border membrane vesicles could be used in the development of an in vitro assay for small-molecule cholesterol absorption inhibitors. For the successful implementation of such an assay, we needed to validate it by synthesizing and examining a number of known ezetimibe analogues for which the corresponding in vivo data were available.

Rabbit brush border membrane vesicles are prepared by shearing the enterocyte brush border membrane region and allowing its reassembly into vesicles with a diameter of 180 nm (Figure 1).[7a,12] Such a preparation can be carried out conveniently in hours, and the vesicles produced can be stored for months. In the experiments, cholesteryl ester is delivered to the brush border membrane vesicles by incorporation into egg phosphatidyl choline small unilamellar vesicles (25 nm diameter) that act as donor particles.[7,13] Direct application of the small-molecule candidates in 1% aqueous dimethylsulfoxide was complicated by their low solubility in water. However, key to the development of the assay was the discovery that these molecules, like the cholesteryl ester, could also be incorporated into the phospholipid small unilamellar vesicles.[14] At a fixed concentration of 9 mol%, corresponding to a nominal concentration of 6 μM, the test substrates were readily incorporated into the vesicles and used in routine inhibition experiments. The cholesterol transfer from donor particles to brush border membrane vesicles was quantified by using radiolabeled [1α,2α(N)-$^3$H]cholesterol oleyl ether (37 Ci mmol$^{-1}$).[15]




***Scheme 1.*** Correlation between in vitro and in vivo activities with various small molecules.

We selected ezetimibe (**1**),[16] β-lactam (±)-**2**,[17,18] and the *O*-glycosides **3**–**5**[19] as convenient benchmark substrates of the in vitro assay, because in vivo results were available for these compounds (Table 1). In the brush border membrane vesicle assay with rabbit intestine, ezetimibe (**1**) was shown to inhibit cholesteryl ester uptake by 16% when the convenient 9-mol% concentration in the vesicles was used. β-Lactam

[*] L. Kværnø, T. Ritter, Prof. Dr. E. M. Carreira
Laboratorium für Organische Chemie
ETH Hönggerberg, HCI H335
8093 Zürich (Switzerland)
Fax: (+41) 1-632-1328
E-mail: carreira@org.chem.ethz.ch
Dr. M. Werder, Prof. Emeritus Dr. H. Hauser
Institute of Biochemistry
ETH Hönggerberg
8093 Zürich (Switzerland)

[**] This research was supported by a CTI grant (6813.2 BTS-LS) and Lipideon AG. L.K. was supported by a fellowship from The Technical University of Denmark. T.R. thanks the Fonds der Chemischen Industrie for a Kekulé Fellowship.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/anie.200460348  

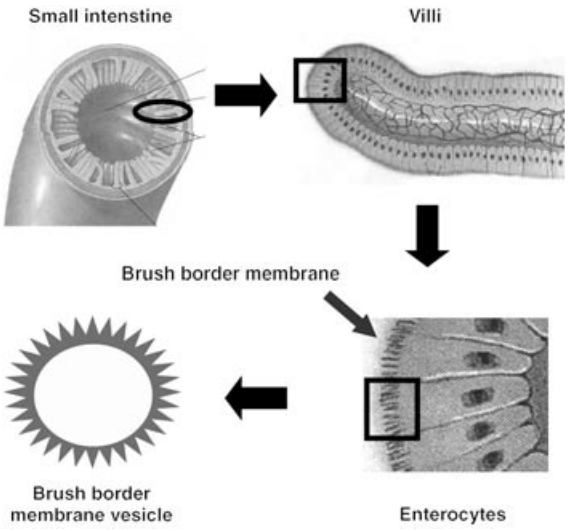
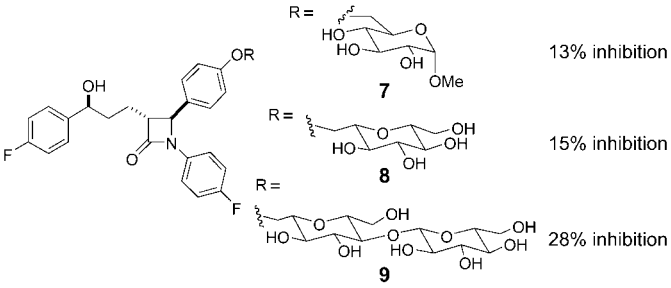



***Figure 1.*** Preparation of brush border membrane vesicles.

(±)-**2** was inactive, a result consistent with the corresponding reported low activity in vivo.[18] Furthermore, the same correlation was observed for the *O*-glycosides as that reported for in vivo studies,[19] wherein cellobioside **5** was significantly more active than glucuronide **3** and glucoside **4**. In the development of ezetimibe (**1**) by using animal studies, the benzylic hydroxy group was shown to be of prime importance for activity.[6c] Likewise in our assay, removal of this hydroxy functionality in **5** to give **6** resulted in a drastic decrease in the in vitro inhibitory activity (3% inhibition for **6** versus 27% for **5**). This molecular editing experiment[21] provides yet another correlation with the in vivo data.

With a validated in vitro assay in hand, we started to address two key issues: 1) the role of glycosylation in inhibitory activity and 2) the use of other ring scaffolds to replace the β-lactam. In vivo, ezetimibe (**1**) has been shown to be rapidly glucuronidated in the intestine to form glucuronide **3** before entering the portal plasma.[22] Rapid metabolic hydrolysis of the *O*-glycosides **3**–**5** and subsequent interconversion between ezetimibe (**1**) and the glucuronide **3** cannot be completely ruled out in the reported animal experiments. Consequently, we designed, synthesized, and examined the structurally related glycosides **7**–**9** with nonhydrolyzable linkages[23] (Scheme 2). These were prepared

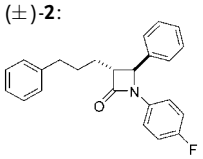
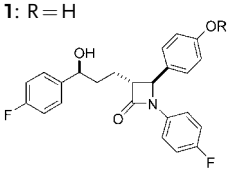
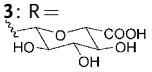
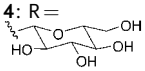
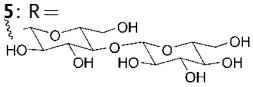
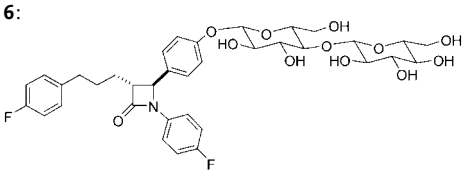



***Scheme 2.*** Nonhydrolyzable glycosides evaluated in the brush border membrane vesicle assay.

by Mitsunobu reactions of **10** with the appropriate primary alcohols, as shown for cellobioside **9** in Scheme 3.

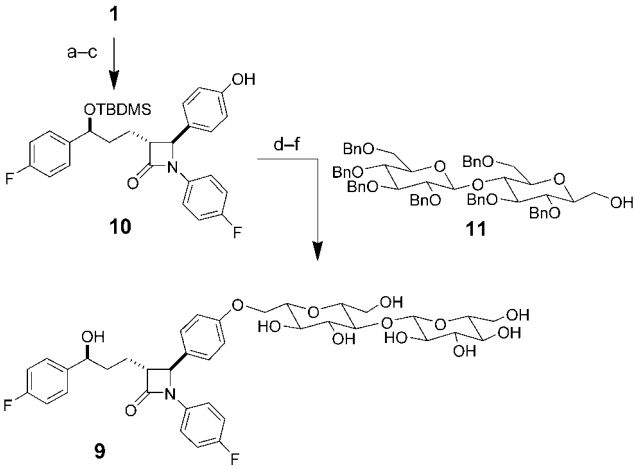
When **7**–**9** were evaluated in the brush border membrane vesicle assay, the glucosides **7** and **8** displayed inhibitions of 13% and 15%, respectively (Scheme 2). The cellobioside **9** (28% inhibition) was clearly more active and was as active an inhibitor as the corresponding *O*-cellobioside **5** (27% inhibition). The activities of the nonhydrolyzable glycosides **7**–**9** indicate that glycosides of ezetimibe (**1**) are indeed capable of being potent cholesterol absorption inhibitors.

***Table 1:*** Activities in the brush border membrane vesicle assay compared to in vivo data.

| Inhibitor | In vivo inhibition [mg kg$^{-1}$ day$^{-1}$][a] | In vitro inhibition [%][b,c] |
|---|---|---|
| (±)-**2**:  | low activity[d][18] | 2 |
| **1**: R = H  | 0.04[6] | 16 |
| **3**: R =  | 0.09[19] | 19 |
| **4**: R =  | 0.08[19] | 14 |
| **5**: R =  | 0.01[19] | 27 |
| **6**:  | – | 3 |

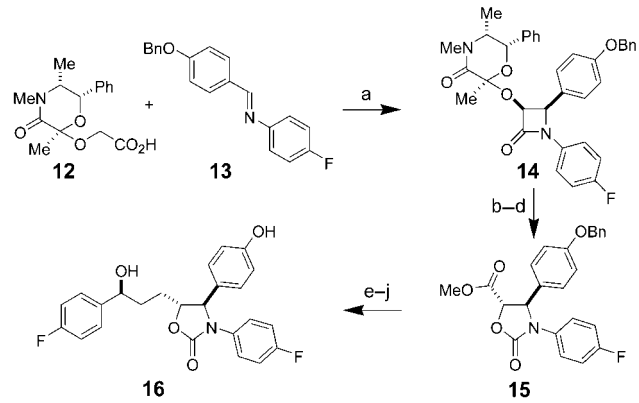
[a] Reported effective dose values for 50% reduction of liver cholesteryl ester level ($ED_{50}$) in the seven-day cholesterol-fed hamster model.[20] [b] Inhibition in the brush border membrane vesicle assay with rabbit intestine at nominal concentrations of 6 μM. [c] Average standard deviations were ±3% inhibition. [d] At 50 mg kg$^{-1}$ day$^{-1}$, no $ED_{50}$ value was determined.




***Scheme 3.*** a) **1**,[16] $Ac_2O$, NaOH, *i*PrOH, 97%; b) TBDMSCl, imid, DMF, 91%; c) $Al_2O_3$ (neutral), 70°C, 83%; d) **11**,[24] 1,1′-(azodicarbonyl)dipiperidine, $Bu_3P$, THF; e) $H_2$, $Pd(OH)_2/C$, EtOAc/EtOH, 21% over two steps; f) HF·pyridine, pyridine, THF, 62%. Bn = benzyl, DMF = *N*,*N*-dimethylformamide, imid = imidazole, TBDMS = *tert*-butyldimethylsilyl, THF = tetrahydrofuran.

The in vitro assay also allowed us to conveniently examine whether the β-lactam is an integral and essential pharmacophore, as concluded in the early development of ezetimibe (**1**),[18,25] or simply a ring scaffold to appropriately position the required substituents. Thus, oxazolidinone **16**, which closely resembles ezetimibe (**1**) with respect to the positioning of the ring substituents,[25] was synthesized in enantiomerically pure form as outlined in Scheme 4. We were pleased to observe that oxazolidinone **16** showed a similar activity (19% inhibition) to ezetimibe (**1**; 16% inhibition) in the brush border membrane vesicle assay. The notable activity for **16** suggests that an oxazolidinone ring scaffold substituted with the appropriate side chains could effectively replace ezetimibe (**1**).




19% inhibition of cholesterol absorption

***Scheme 4.*** a) **12**,[26] **13**,[27] $(Cl_3CO)_2CO$, $Et_3N$, 0°C→RT, 75%, d.r. = 3:2; b) THF/$H_2O$, reflux, 50%; c) NaOMe, MeOH; d) $(Cl_3CO)_2CO$, $iPr_2NEt$, DMAP, $CH_2Cl_2$, −78°C→23°C, 73% over two steps; e) $NaBH_4$, EtOH; f) $(COCl)_2$, DMSO, $Et_3N$, $CH_2Cl_2$, −78°C; g) $pFC_6H_4COCH{=}PPh_3$, $CH_2Cl_2$, −78°C→23°C, 82% over three steps; h) $H_2$, Pd/C, EtOH; i) (*R*)-CBS,[28] $BH_3{\cdot}Me_2S$, $CH_2Cl_2$, −20→0°C; j) $H_2$, Pd/C, EtOH, 57% over three steps. (*R*)-CBS = *R*-tetrahydro-1-methyl-3,3-diphenyl-1*H*,3*H*-pyrrolo(1,2-C)(1,3,2)oxazaborolidine, DMAP = 4-dimethylaminopyridine, DMSO = dimethylsulfoxide.

In summary, we have used brush border membrane vesicles in the first in vitro assay to evaluate small-molecule cholesterol absorption inhibitors. In comparison with published in vivo data of identical and closely related compounds, consistent in vitro results were obtained. These indicate that both ezetimibe (**1**) and a variety of ezetimibe glycosides are all potent cholesterol absorption inhibitors. The salient feature of the assay is that it permits rapid identification of other useful ring scaffolds for the synthesis of novel cholesterol absorption inhibitors, as exemplified by the oxazolidinone **16**. It should thus be possible to replace tedious, expensive, and lengthy animal experiments by convenient evaluation in the brush border membrane vesicle assay when investigating diverse libraries of small-molecule cholesterol absorption inhibitors.

Received: April 16, 2004

**Keywords:** cholesterol · inhibitors · medicinal chemistry · membranes

[1] Heart Disease and Stroke. Statistics—2003 Update. Available from The American Heart Association at: http://www.americanheart.org/downloadable/heart/1059017971148 2003HDSStatsBookREV7-03.pdf.
[2] T. Sudhop, K. von Bergmann, *Drugs* **2002**, *62*, 2333.
[3] E. Bruckert, *Cardiology* **2002**, *97*, 59.
[4] D. J. Maron, S. Fazio, M. F. Linton, *Circulation* **2000**, *101*, 207.
[5] Euroaspire II Study Group, *Eur. Heart J.* **2001**, *22*, 554.
[6] a) FDA Drug Approvals List [online]: http://www.fda.gov/cder/foi/label/2002/21445lbl.pdf; b) J. W. Clader, *J. Med. Chem.* **2004**, *47*, 1; c) S. B. Rosenblum, T. Huynh, A. Afonso, H. R. Davis, N. Yumibe, J. W. Clader, D. A. Burnett, *J. Med. Chem.* **1998**, *41*, 973.
[7] a) H. Thurnhofer, H. Hauser, *Biochemistry* **1990**, *29*, 2142; b) S. Compassi, M. Werder, D. Boffelli, F. E. Weber, H. Hauser, G. Schulthess, *Biochemistry* **1995**, *34*, 16473; c) S. Compassi, M. Werder, F. E. Weber, D. Boffeli, H. Hauser, *Biochemistry* **1997**, *36*, 6643; d) G. Schulthess, S. Compassi, D. Boffelli, M. Werder, F. E. Weber, H. Hauser, *J. Lipid Res.* **1996**, *37*, 2405; e) H. Hauser, J. H. Dyer, A. Nandy, M. A. Vega, M. Werder, E. Bieliauskaite, F. E. Weber, S. Compassi, A. Gemperli, D. Boffelli, E. Wehrli, G. Schulthess, M. C. Phillips, *Biochemistry* **1998**, *37*, 17843; f) M. Werder, C. H. Han, E. Wehrli, D. Bimmler, G. Schulthess, H. Hauser, *Biochemistry* **2001**, *40*, 11643.
[8] a) S. F. Cai, R. J. Kirby, P. N. Howles, D. Y. Hui, *J. Lipid Res.* **2001**, *42*, 902; b) L. Cai, E. R. M. Eckhardt, W. Shi, Z. Zhao, M. Nasser, W. J. S. de Villiers, D. R. van der Westhuyzen, *J. Lipid Res.* **2004**, *45*, 253; c) S. W. Altmann, H. R. Davis, X. R. Yao, M. Laverty, D. S. Compton, L. J. Zhu, J. H. Crona, M. A. Caplen, L. M. Hoos, G. Tetzloff, T. Priestley, D. A. Burnett, C. D. Strader, M. P. Graziano, *Biochim. Biophys. Acta* **2002**, *1580*, 77; d) E. Levy, D. Menard, I. Suc, E. Delvin, V. Marcil, L. Brissette, L. Thibault, M. Bendayan, *J. Cell Sci.* **2004**, *117*, 327; e) D. Jourdheuil-Rahmani, M. Charbonnier, N. Domingo, F. Luccioni, H. Lafont, D. Lairon, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 390; f) B. Play, S. Salvini, Z. Haikal, M. Charbonnier, A. Harbis, M. Roussel, D. Lairon, D. Jourdheuil-Rahmani, *Biochem. Biophys. Res. Commun.* **2003**, *310*, 446.

[9] A. van Bennekum, M. Werder, S. T. Thuahnai, C.-H. Han, D. L. Williams, P. Wettstein, G. Schulthess, M. C. Phillips, H. Hauser, Biochemistry **2004**, submitted.
[10] S. W. Altmann, H. R. Davis, L. J. Zhu, X. R. Yao, L. M. Hoos, G. Tetzloff, S. P. N. Iyer, M. Maguire, A. Golovko, M. Zeng, L. Q. Wang, N. Murgolo, M. P. Graziano, *Science* **2004**, *303*, 1201.
[11] E. J. Smart, R. A. De Rose, S. A. Farber, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 3450.
[12] H. Hauser, K. Howell, R. M. C. Dawson, D. E. Bowyer, *Biochim. Biophys. Acta* **1980**, *602*, 567.
[13] Cholesteryl ester, instead of free cholesterol, was chosen for the standard inhibition experiments, since passive diffusion is negligible for cholesteryl ester.[7e]
[14] The incorporation into vesicles was shown to be quantitative for representative inhibitors by gel filtration of the vesicles according to established procedures (see G. Schulthess, S. Compassi, M. Werder, C. H. Han, M. C. Phillips, H. Hauser, *Biochemistry* **2000**, *39*, 12623, and references therein) followed by reversed-phase HPLC analysis.
[15] Radiolabeled cholesterol oleyl ether was used as a nonhydrolyzable analogue of cholesterol oleyl ester, since both have been shown to exhibit identical brush border membrane uptake kinetics.[7b]
[16] G. Z. Wu, Y. S. Wong, X. Chen, Z. X. Ding, *J. Org. Chem.* **1999**, *64*, 3714.
[17] M. Browne, D. A. Burnett, M. A. Caplen, 0L. Y. Chen, J. W. Clader, M. Domalski, S. Dugar, P. Pushpavanam, R. Sher, W. Vaccaro, M. Viziano, H. R. Zhao, *Tetrahedron Lett.* **1995**, *36*, 2555.
[18] J. W. Clader, D. A. Burnett, M. A. Caplen, M. S. Domalski, S. Dugar, W. Vaccaro, R. Sher, M. E. Browne, H. R. Zhao, R. E. Burrier, B. Salisbury, H. R. Davis, *J. Med. Chem.* **1996**, *39*, 3684.
[19] W. D. Vaccaro, H. R. Davis, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 313.
[20] a) D. A. Burnett, M. A. Caplen, H. R. Davis, R. E. Burrier, J. W. Clader, *J. Med. Chem.* **1994**, *37*, 1733; b) R. Schnitzerpolokoff, D. Compton, G. Boykow, H. Davis, R. Burrier, *Comp. Biochem. Physiol. Part A* **1991**, *99*, 665.
[21] a) A. M. Rouhi, *Chem. Eng. News* **2003**, *81*, 104; for recent examples of successful molecular editing of epothilones, see: b) T. C. Chou, H. J. Dong, A. Rivkin, F. Yoshimura, A. E. Gabarda, Y. S. Cho, W. P. Tong, S. J. Danishefsky, *Angew. Chem.* **2003**, *115*, 4910; *Angew. Chem. Int. Ed.* **2003**, *42*, 4761; c) K. C. Nicolaou, P. K. Sasmal, G. Rassias, M. V. Reddy, K. H. Altmann, M. Wartmann, A. O'Brate, P. Giannakakou, *Angew. Chem.* **2003**, *115*, 3639; *Angew. Chem. Int. Ed.* **2003**, *42*, 3515.
[22] M. van Heek, C. Farley, D. S. Compton, L. Hoos, K. B. Alton, E. J. Sybertz, H. R. Davis, *Br. J. Pharmacol.* **2000**, *129*, 1748.
[23] W. D. Vaccaro, R. Sher, H. R. Davis, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 35.
[24] S. J. Spak, O. R. Martin, *Tetrahedron* **2000**, *56*, 217.
[25] S. Dugar, M. P. Kirkup, J. W. Clader, S. I. Lin, R. Rizvi, M. E. Snow, H. R. Davis, S. W. McCombie, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2947.
[26] B. A. Shinkre, V. G. Puranik, B. M. Bhawal, A. Deshmukh, *Tetrahedron: Asymmetry* **2003**, *14*, 453.
[27] T. Kambara, K. Tomioka, *J. Org. Chem.* **1999**, *64*, 9282.
[28] a) E. J. Corey, R. K. Bakshi, S. Shibata, *J. Am. Chem. Soc.* **1987**, *109*, 5551; b) E. J. Corey, R. K. Bakshi, S. Shibata, C. P. Chen, V. K. Singh, *J. Am. Chem. Soc.* **1987**, *109*, 7925.