Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 1639–1642

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis of a Biotin-Tagged Photoaffinity Probe of 2-Azetidinone Cholesterol Absorption Inhibitors

Wendelin Frick, Andrea Bauer-Schäfer, Jochen Bauer, Frank Girbig, Daniel Corsiero, Hubert Heuer and Werner Kramer*

Aventis Pharma Deutschland GmbH, Disease Group Metabolic Diseases Industriepark Höchst, Building G 879 D-65926, Frankfurt am Main, Germany

Received 5 November 2002; accepted 17 January 2003

Abstract—The design and synthesis of a biotin-tagged photoreactive analogue C-4 of the cholesterol absorption inhibitor Ezetimibe is described. Photoaffinity labeling of intestinal brush border membrane vesicles with C-4 and subsequent streptavidin–biotin chromatography leads to selective extraction of a 145 kDa integral membrane protein as the molecular target for cholesterol absorption inhibitors.

Intestinal cholesterol absorption is a major regulator of serum cholesterol levels.[1] Consequently, inhibition of intestinal cholesterol absorption is a novel pharmacological mechanism to regulate serum cholesterol levels. Specific inhibitors for cholesterol absorption found accidentally have proven their clinical efficacy.[2,3] The known cholesterol absorption inhibitors belong to two different chemical classes, the 2-azetidinones **1**,**2** and the sterol glycosides **3**,**4** (Fig. 1), both classes showing profound structure–activity relationships.[4,5] Despite their pharmacological efficacy, the molecular mode of action of these compounds and the mechanism of intestinal cholesterol absorption is still unknown. For the discovery of novel cholesterol absorption inhibitors and optimisation of their structure–activity relationships the availability of the molecular target for cholesterol absorption inhibitors is a prerequisite. By photoaffinity labelling with radioactively labelled photoreactive 2-azetidinone cholesterol absorption inhibitors[6,7] we succeeded in the identification of an integral 145-kDa membrane protein as the molecular target for cholesterol absorption inhibitors localized in the brush border membrane of absorptive enterocytes from the rabbit small intestine.[6] An efficacious purification procedure for the 145-kDa protein is necessary to determine its amino acid sequence and to clone the protein. We

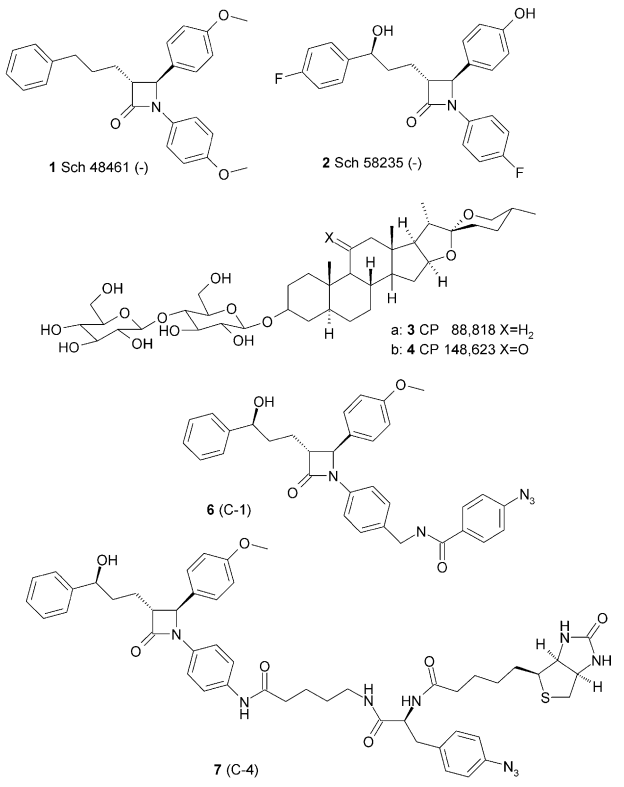

Figure 1. Structures of known cholesterol absorption inhibitors (**1**–**4**) and structures of two photoreactive analogous 2-azetidiones (**C-1** and **C-4**).

*Corresponding author. Tel.: +49-69-305-3557; fax +49-69-305-13333; e-mail: werner.kramer@aventis.com

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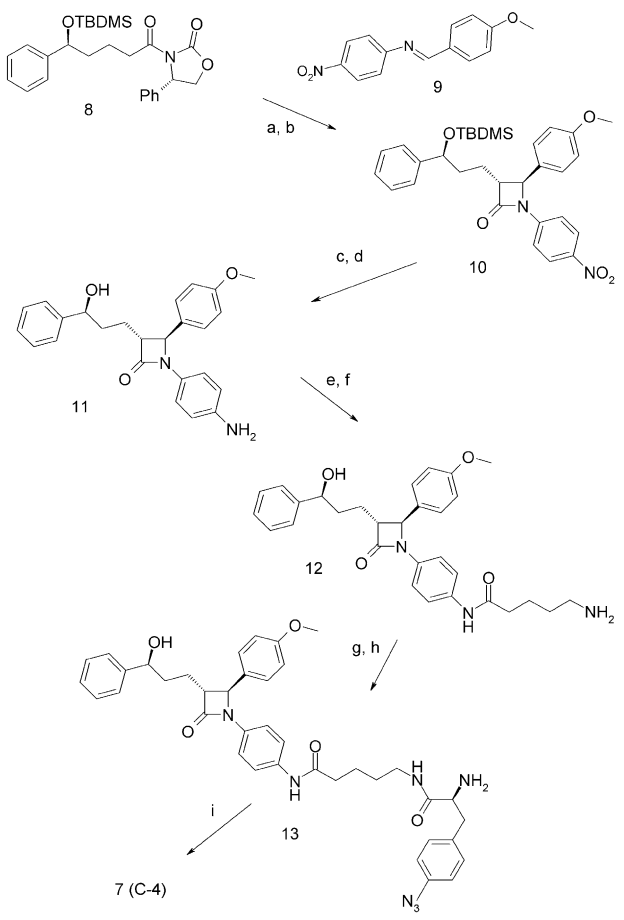
doi:10.1016/S0968-0896(03)00047-6

therefore aimed to extract the 145 kDa from solubilized membrane proteins by streptavidin–biotin affinity chromatography using a photoreactive biotinylated derivative of the highly efficacious cholesterol absorption inhibitor Sch 58235 (Ezetimibe) (Fig. 1). Structure–activity relationships of 2-azetidinone cholesterol absorption inhibitors[4] revealed that in the *para*-position of the *N*-phenylring rather bulky groups such as azidobenzoyl[6,7] or even fluorescent reporter groups[8] can be introduced conserving the pharmacological activity to inhibit intestinal cholesterol absorption. We therefore decided to use this position for the attachment of photoreactive groups to allow a covalent crosslinking of the probe to its binding protein as well as of the biotinyl group for the subsequent streptavidin extraction of the photocrosslinked target protein(s) (Scheme 1).

Synthesis of Biotinylated Photoaffinity Probe C-4

Our synthesis of the biotin-labeled photoaffinity compound **7** (C-4) is based on an asymmetric synthesis of ezetimibe (Sch 58235). The enatiomerically pure 5′-(*S*)-*t*-butyl-dimethyl-silyloxy-5′-(phenyl) valeroyl-5-(*S*)-phenyloxazolidinone **8** was treated with Hünig's base, trimethylsilyl chloride and titanium tetrachloride to give the intermediate β-amino acyloxazolidinone as an 2:1 mixture of diastereomers. This mixture of diastereomers was used for the cyclization reaction with bis-trimethylsilyl acetamide (BSA) and catalytic amount of tetrabutylammonium fluoride (TBAF) to get azetidinone **10**. The azetidinone **10** was further used as a diastereomeric mixture (it was not possible to separate this mixture of diastereomers at any step, so the mixture was used for the whole synthesis. In the reaction scheme 1 only the major diastereomer is shown). For reduction of the nitrogroup of azetidinone **10**, the compound was hydrogenated with palladium on charcoal (10% Pd). Removal of the silylprotecting group was carried out in an homogeneous mixture of tetrahydrofurane and aqueous 2 N HCl at room temperature. The anilide **11** was then coupled twice, first with Fmoc-protected 5-aminovalerianic acid and then with Fmoc-*p*-azido-phenyl alanine. For this reaction we have used *O*-((ethoxycarbonyl) cyanomethyleneamino),*N*,*N*,*N*,*N*-tetramethyluronium-tetraflouroborate (TOTU) as coupling reagents. Finally the photolabeled compound **13** was coupled with D-biotin-*N*-succinimidyl ester which is commercial available from Bachem (Germany) to get the double labeled (with biotin and azido group) compound **7** (C-4) as a 2:1 mixture of diastereomers.[9,10]



Scheme 1. Synthesis of biotin-labeled photoaffinity probe **C-4**: (a) DIPEA, TMSCl, $TiCl_4$, CH_2Cl_2 (74%); (b) BSA, MTB-ether, TBAF (cat) (70%); (c) H_2, Pd/C, 2 h, 5 bar, ethyl acetate (86%); (d) aqueous 2 N HCl, THF, 2 h, rt; (e) 5-(Fmoc-amino)-valerianic acid, TOTU, ethyl (hydroxyimino)-cyanoacetate, NEM (all from Fluka), DMF, 1 h, rt (41%); (f) $NHEt_2$, DMF, 1 h, rt (56%); (g) Fmoc-*p*-azido-Phe-OH (Bachem), TOTU, ethyl (hydroxyimino)-cyanoacetate, NEM (all from Fluka), DMF, 1 h, rt (82%); (g) $NHEt_2$, DMF, 1 h, rt (19%); (i) D-biotin-*N*-succinimidyl ester (Bachem), DMF, 1 h, rt (55%).

Biological Characteristics of the Biotinylated Photoaffinity Probe for 2-Azetidinone Cholesterol Inhibitors

In order to evaluate whether the introduction of the photolabile and the biotinyl reporter groups into the Ezetimibe molecule would biologically be tolerated, the compound was administered to NMRI mice and its effect on intestinal cholesterol absorption was measured using the Zilversmit/Hughes method[11] as described.[6] As with Ezetimibe, intestinal cholesterol absorption as measured by an increased fecal excretion and a decreased hepatic content of the administered radioactively labeled cholesterol was inhibited by the probe (Table 1); its biological activity was, however, lower than that of Ezetimibe indicating a negative influence of the attached reporter groups on pharmacological efficacy.

Photoaffinity labeling of the 145 kDa protein by the radiolabeled 2-azetidinone cholesterol absorption inhibitor C-1 was concentration-dependently and specifically inhibited by the presence of C-4 indicating that the biotin-tagged Ezetimibe photoaffinity probe C-4 specifically interacts with the 145 kDa binding protein for cholesterol absorption inhibitors. Consequently, C-4 should be suited for purification of the Ezetimibe target protein by streptavidin–biotin chromatography. After photoaffinity labeling of rabbit small intestinal brush border membrane vesicles with C-4 membrane proteins were solubilized and streptavidin–agarose beads were added. After extensive washing, proteins retained by the streptavidin matrix were eluted either with buffer containing 6 mM biotin or under denaturating conditions with SDS-

Table 1. In vivo activity of the biotin-tagged photoreactive cholesterol absorption inhibitor C-4 in NMRI mice[6,11]

Compd	^{14}C-Cholesterol excretion in feces (nCi)	^{14}C-Cholesterol uptake by the liver (nCi)
Control	280	78
Ezetimibe (0,1 mg/mouse)	480 (+ 71%)	15 (−81%)
Ezetimibe (1 mg/mouse)	490 (+ 75%)	4 (−95%)
C-4 (1 mg/mouse)	370 (+ 32%)	38 (−51%)

0.5 mL of a solution of the indicated amount of the cholesterol absorption inhibitors in 0.5% methylcellulose/5% Solutol® were administered by gavage to male NMRI mice (four animals per group) followed by 0.25 mL of Intralipid® solution containing 0.34 μCi [^{14}C] cholesterol per animal. After 24 h, the amount of radioactivity in feces and liver was determined. (The data represent the radioactivity found in feces and liver of the four animals in each treatment group).

solutions.[12] SDS-gel electrophoresis of the eluates revealed that a 145 kDa protein was selectively extracted by the streptavidin beads (Fig. 2, lane B). No 145 kDa protein could be detected if ultraviolet irradiation was omitted (Fig. 2, lane A) thereby preventing a covalent attachment of the photolabile ligand analogue to its specific binding protein(s). It is remarkable to detect such a clear protein band after photoaffinity labelling since only a small percentage of the activated nitrene of the ligand crosslinks with amino acid moieties in the binding site of the target protein. Furthermore, only from brush border membranes isolated from the organs where intestinal cholesterol absorption occurs, the small intestine, the 145 kDa protein could be extracted, whereas from stomach, cecum, colon, rectum, kidney, liver or fat tissue plasma membranes no 145 kDa protein could be enriched.

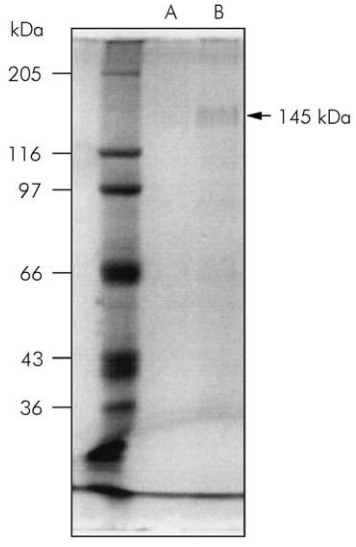


Figure 2. Photoaffinity labelling of rabbit ileal brush border membrane vesicles with C-4 and following streptavidin–biotin extraction. 200 μg of rabbit ileal brush border membrane vesicles were incubated with 200 μM of photoaffinity probe C-4 without (A) or with UV-irradiation (B). After solubilisation of membrane proteins, the proteins containing the biotin-tagged cholesterol absorption inhibitor C-4 were extracted with streptavidin–agarose beads. Bound proteins were released with 6 mM biotin and subsequently analysed by SDS-gel electrophoresis and staining with Coomassie-Blue R-250.

In conclusion, the biotinylated photoaffinity probe C-4 of 2-azetidinone cholesterol absorption inhibitors is well suited to enable an efficient enrichment and purification of the target protein for cholesterol absorption inhibitors. Final purification, sequencing and cloning of the 145 kDa protein will improve our understanding of the mechanism of intestinal cholesterol absorption and will help to identify novel cholesterol absorption inhibitors.

Acknowledgements

The authors greatly thank Meike Scharnagl, Susanne Nicolaus and Klaus Bock for excellent assistance.

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9. All compounds were characterized by ^{1}H NMR, and the purity by LC–MS.
10. **Synthesis of Compound C-4 (7). 3-{5-(*tert*-Butyl-dimethyl-silanyloxy)-2-[(4-methoxy-phenyl)-(4-nitro-phenylamino)-methyl]-5-phenyl-pentanoyl}-4-phenyl-oxazolidin-2-on (10).** To 5.4 g (12.0 mmol) of compound 8 and 6.2 g (24 mmol) of compound 9 in 135 mL of methylenchloride 8 mL of diisopropylethylamine is added at 10 °C and 4.8 mL of trimethylsilylchloridie is added dropwise. After 1 h, 14 mL of a 1 molar solution of titantetrachloride in methylenchloride is added dropwise at −10 °C. It is stirred for 3 h at −10 °C and further 12 h at −30 °C stored without stirring. Afterwards 8 mL of acetic acid and 140 mL of a 7% aqueous solution of tartaric acid is added and stirred for further 2 h at room temperature. After addition of 50 mL of a 20% aqueous solution of sodium hydrogensulfite it is stirred for another 1 h and extracted with methylenchloride. The organic phase is dried by magnesiumsulfate, evaporated and purified by chromatography on silica/ethylacetate/heptane = 1/3→1/1. 6.3 g (74%) of titan reaction product is obtained in form of a light yellow solid compound: $C_{40}H_{47}N_3O_7Si$ (709.92) MS (ESI$^+$) 710 ($M+H^+$). A mixture comprising 6.1 g (8.6 mmol) of titan reaction product, 7.3 mL of bistrimethylsilylacetamide, 0.5 g of tetrabutylammoniumfluorid and 100 mL of *tert*-butylmethylether is

stirred under an argon atmosphere for 10 h at room temperature. After finishing of the reaction 5 mL of acetic acid are added slowly by cooling with ice and evaporated. The residue is separated by chromatography on silica gel (ethylacetate/heptan = 1/2). 3.3 g (70%) of product 10 is obtained in form of a light yellow compound in solid form: $C_{31}H_{38}N_2O_5Si$ (546.74) MS (ESI$^+$) 547.3 (M + H$^+$). **1-(4-Amino-phenyl)-3-(3-hydroxy-3-phenyl-propyl)-4-(3-methoxy-phenyl)-azetidin-2-on (11).** A reaction is performed with 3.0 g (5.5 mmol) of product 10 in 50 mL ethylacetate and 1.0 g of palladium charcoal 10% for 2 h at 5 bar of a hydrogen atmosphere using an autoclave. The reaction solution is filtrated, evaporated and separated by chromatography on silica gel (methylenchloride/methanol = 10/1). 2.4 g (86%) of the anilide is obtained in form of a colourless compound in solid form: $C_{31}H_{40}N_2O_3Si$ (516.76) MS (ESI$^+$) 517.4 (M + H$^+$). 15 mL of 2 N aqueous hydrochloric acid are added to 2.3 g of the anilide in 20 mL of tetrahydrofuran and stirred for 2 h. An aqueous solution of sodium hydrogencarbonate is added to the reaction and extracted by ethylacetate. The organic phase is dried by magnesium sulfate, evaporated and purified by chromatography on silica gel (ethylacetate/heptan = 1/1→1/0). 1.1 g of product 11 is obtained in form of a colorless compound in solid form: $C_{25}H_{26}N_2O_3$ (402.50) MS (ESI$^+$) 403.2 (M + H$^+$). **5-Amino-pentanoic acid-{4-[3-(3-hydroxy-3-phenyl-propyl)-2-(4-methoxy-phenyl)-4-oxo-azetidin-1-yl]-phenyl}-amide (12).** 0.8 g (2,0 mmol) of product 11 and 1.35 g (4.0 mmol) of 5-(Fmoc-amino)-valerianic acid (Fluka) are solved in 15 mL of DMF (dimethylformamide). Stepwise is added the following: 4.8 g of TOTU (Fluka), 1.6 g of oxime (hydroxyimino-cyanoacetic acid-ethylester; Fluka) and 5.5 mL of NEM (4-ethyl-morpholine). After 1 h at room temperature, the reaction is diluted with 100 mL of ethylacetat and washed for three times with water. The organic phase is dried by $MgSO_4$, filtrated and evaporated. The residue is purified by flash chromatography (ethylacetate/*n*-heptan 2:1). 0.58 g (41%) of coupling product is obtained in form of a amorphic solid compound: $C_{45}H_{45}N_3O_6$ 723.8) MS (ESI$^+$) 724.4 (M + H$^+$). 570 mg (0.78 mmol) of the coupling product and 0.8 mL of diethylamine are solved in 5 mL of DMF (dimethylformamide). It is evaporated after 1 h at room temperature. The residue is purified by flash chromatography (methylenchloride/methanol/concd ammonia 30:10:3) 220 mg (56%) of product 12 is obtained in form of an amorphic solid compound: $C_{30}H_{35}N_3O_4$ (501.63) MS (ESI$^+$) 502.3 (M + H$^+$). **5-[2-Amino-3-(4-azido-phenyl)-propionylamino]-pentanoic acid{4-[3-(3-hydroxy-3-phenyl-propyl)-2-(4-methoxy-phenyl)-4-oxo-azetidin-1-yl]-phenyl}-amid (13).** 200 mg (0.40 mmol) of product 12 and 340 mg (0.79 mmol) of Fmoc-*p*-azido-Phe-OH (Bachem) are solved in 4 mL DMF (dimethylformamide) and reacted according to production of product 12. 42 mg of compound 13 is obtained in form of an amorphic solid compound: $C_{39}H_{43}N_7O_5$ (689.82) MS (ESI$^+$) 690.3 (M + H$^+$). **5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid-[2-(4-azido-phenyl)-1-(4-{4-[3-(3-hydroxy-3-phenyl-propyl)-2-(4-methoxy-phenyl)-4-oxo-azetidin-1-yl]-phenylcarbamoyl}-butylcarbamoyl)-ethyl]-amid (7).** 40 mg (0.058 mmol) of compound 12 and 60 mg of D-biotinyl-*N*-hydroxysuccinimide (Bachem) are solved in 0.5 mL DMF (dimethylformamide). It is evaporated after 1 h at room temperature. The residue is purified by flash chromatography (methylenchloride/methanol/concd ammonia 30:5:1). 29 mg (55%) of compound 7 is obtained in form of an amorphic solid compound as a 2:1 mixture of diastereomers: $C_{49}H_{57}N_9O_7S$ (916.12) MS (ESI$^+$) 916.6 (M + H$^+$).

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12. **Photoaffinity labeling and streptavidin-biotin affinity chromatography.** Rabbit ileal brush border membrane vesicles (200 μg of protein) prepared as described[13] were photolabelled with 200 μM of C-4 in 10 mM Tris/Hepes buffer (pH 7.4)/100 mM NaCl/100 mM mannitol by ultraviolet irradiation for 30 s at 254 nm in a Rayonet RPR 100 photochemical reactor equipped with 4 RPR 2537 Å lamps. After washing membrane proteins were solubilized with 250 μL of 10 mM Tris/Hepes buffer (pH 7.4)/75 mM KCl/5 mM $MgCl_2$/1 mM EGTA/1 mM DTT/1% (w/v) *n*-octylglucoside/1% (w/v) Triton X-100/1 mM Pefabloc and kept at 4 °C for 60 min. After centrifugation the supernatant containing the solubilized membrane proteins was added to 50 μL of streptavidin-agarose beads and kept under stirring at 4 °C for 2 h. After centrifugation, the beads were washed three times with 250 μL of 10 mM Tris/Hepes buffer (pH 7.4)/300 mM mannitol/1% *n*-octylglucoside/4 mM PMSF/4 mM iodoacetamide/4 mM EDTA at 4 °C. Streptavidin-bound proteins were eluted with 250 μL of the above buffer containing 6 mM biotin and stirring for 60 min at 4 °C. After centrifugation, proteins were precipitated with chloroforme/methanol and analysed by SDS gel electrophoresis..[13]

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