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(54) Titre : PROTEINE ISSUE DE L'INTESTIN D'ANIMAUX VERTEBRES, QUI ABSORBE LE CHOLESTEROL,
UTILISATION DE LADITE PROTEINE POUR IDENTIFIER DES INHIBITEURS DU TRANSPORT INTESTINAL DU
CHOLESTEROL

(54) Title: VERTEBRATE INTESTINAL PROTEIN WHICH ABSORBS CHOLESTEROL, AND THE USE OF THIS
PROTEIN FOR IDENTIFYING INHIBITORS OF INTESTINAL CHOLESTEROL TRANSPORT

(57) **Abrégé/Abstract:**

The invention relates to a vertebrate intestinal protein which absorbs cholesterol. It was possible to identify the protein by means of high-affinity crosslinking compounds. The invention further relates to the use of this protein for carrying out a method for identifying a compound which inhibits cholesterol transport in the intestine.



Abstract:

The invention relates to a vertebrate intestinal protein which absorbs cholesterol. It was possible to identify the protein by means of high-affinity crosslinking compounds. The invention further relates to the use of this protein for carrying out a method for identifying a compound which inhibits cholesterol transport in the intestine.

Vertebrate intestinal protein which absorbs cholesterol, and the use of this protein for identifying inhibitors of intestinal cholesterol transport.

- 5 The invention relates to a vertebrate intestinal protein which absorbs cholesterol. The protein is characterized by means of high-affinity crosslinking compounds. The invention further relates to the use of the protein for carrying out a method for identifying a compound which inhibits cholesterol transport in the intestine.
- 10 In humans, on average about 50% of the cholesterol is present in the lumen of the intestine. The intraluminal cholesterol originates mainly from the diet and from the bile. About 2 g of cholesterol a day is discharged from the bile. The intestinal cholesterol absorption depends greatly on the presence of bile salts. Thus the effect of administration of inhibitors of the reuptake of bile salts or of bile salt sequestrants
- 15 is to inhibit intestinal cholesterol absorption.

Inhibition of intestinal cholesterol absorption is an important aim of the treatment of lipid disorders, atherosclerosis and cardiovascular disorders. The prevailing opinion amongst experts is that intestinal cholesterol absorption takes place by

20 physicochemical diffusion.

A number of observations in connection with cholesterol transport which indicate that a protein is involved are known. Intestinal cholesterol absorption is subject to great individual variability. Biochemical data from in vitro experiments indicate that proteins

25 are involved in cholesterol exchange between small unilamellar vesicles and the brush border vesicles of the intestine. It was possible to observe large differences in the intestinal absorption of plant sterols such as β -sitosterol and campesterol, which differ only in a methyl group (β -sitosterol) and an ethyl group (campesterol). In humans, β -sitosterol showed inter alia an inhibition of cholesterol absorption. There

30 are two highly active classes of compounds which inhibit intestinal cholesterol absorption on luminal administration. The compounds are, on the one hand, compounds derived from saponin, such as tiqueside and pamaqueside, and on the other hand certain derivatives of 2-azetidinones. Derivatives of 2-azetidinones as

inhibitors of cholesterol absorption are described in Clader et al., J. Med. Chem. 39, 3684-3693, 1996. For the purposes of this invention, absorption is intended to mean attachment of a substance to a protein and transport of this substance with the aid of this protein.

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Several proteins have to date been thought to be associated with cholesterol absorption. However, as yet no protein unambiguously involved in cholesterol absorption in the intestine has been described. This leads to a number of disadvantages in the search for a rational procedure for identifying specific inhibitors of intestinal cholesterol absorption. This is of particular importance also because inhibitors of intestinal cholesterol absorption are especially suitable for the treatment of all types of lipid disorders, atherosclerosis and cardiovascular disorders. Cholesterol is distributed uniformly in all cells both in the lipid bilayer of the membranes and in lipid vesicles. Transport measurements with vesicles or cells are therefore very complex and subject to interference.

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It is therefore an object of the present invention to provide a protein which is involved in intestinal cholesterol absorption.

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The invention therefore relates to a cholesterol-absorbing protein from the intestinal tissue of a mammalian organism, obtainable by the following process steps:

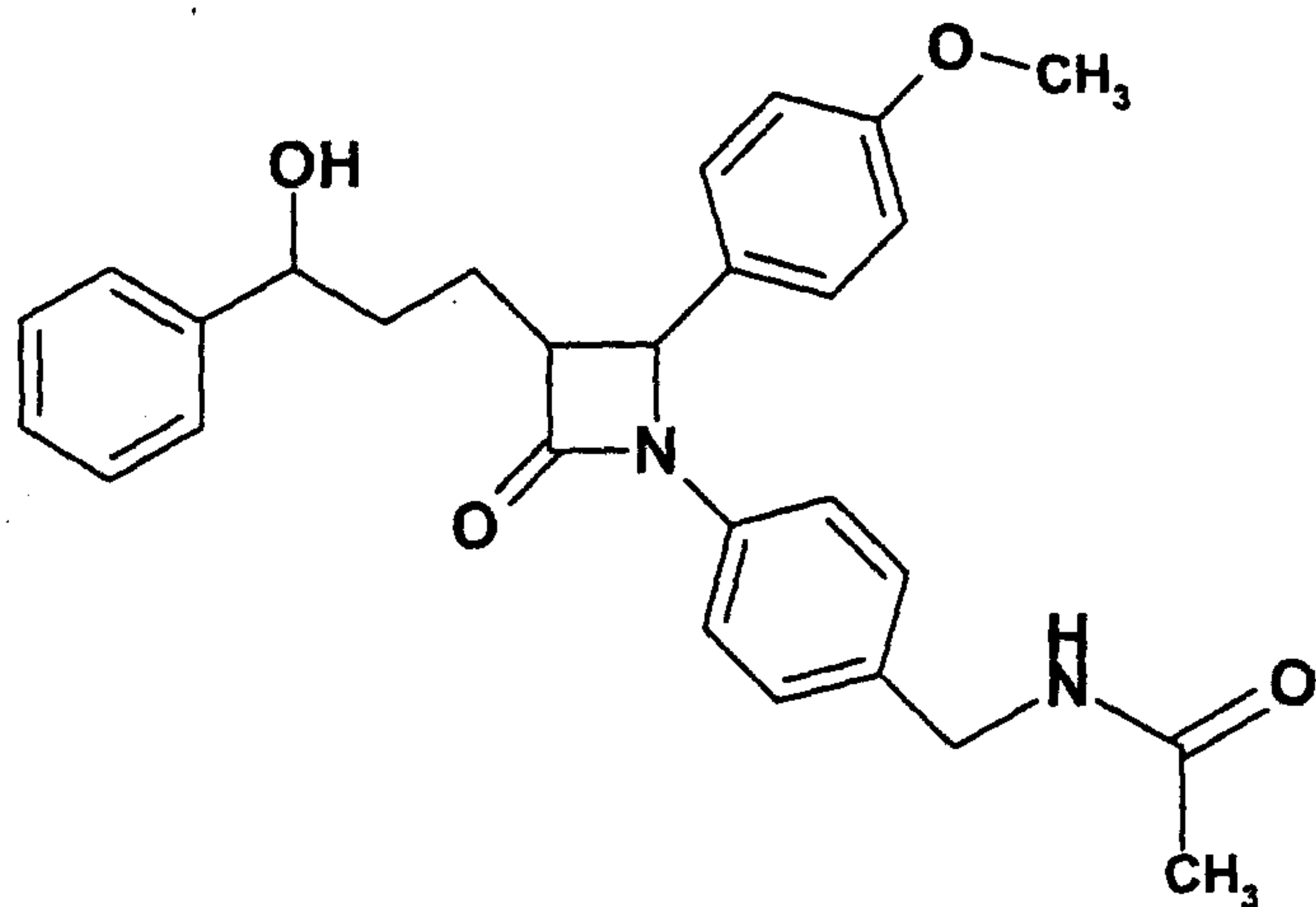
- a) provision of intestinal cells or parts of these intestinal cells of a mammalian organism,
- 25 b) provision of a radiolabeled compound which acts as inhibitor of intestinal cholesterol absorption and contains a photolabile group,
- c) contacting the intestinal cells or parts of these intestinal cells from a) with a compound from b)
- d) irradiation of the mixture from c) with UV light,
- 30 e) disruption of the cells after irradiation as in d),
- f) fractionation of the components of the disrupted cells after disruption as in e),
- g) detection, after fractionation as in f), of a protein which contains a bound compound from b).

The provision of intestinal cells can take place, for example, by dissection of the intestine from animals and subsequent purification, enzymatic disruption of the connective tissue and suspension of single cells in isotonic buffer solutions. Intestinal tissues suitable for the provision of intestinal cells are, inter alia, the corresponding parts of animals remaining after slaughtering. Intestinal cells can also be provided from human intestinal tissue after parts of the intestine have been obtained at operation. The intestinal cells can also consist of intestinal cell cultures provided for the purpose of the invention by application of cell culture techniques. Parts of intestinal cells may be organelles of the intestinal cells. Organelles are, in particular, membranes of the intestinal cells. Membranes of the intestinal cells can be obtained by differential centrifugation after disruption of these cells. Parts of intestinal cells are, in particular, also protein fractions. Provision of intestinal cells or parts of intestinal cells are familiar to the skilled worker, in this case a biochemist. Reference is made in particular to the following textbooks:

"Basic Cell Culture, A practical approach, IRL press (1994), Editor: J.M. Davis" and "Current Protocols in Protein Science, John Wiley & Sons (2000), Editors: J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher, P. T. Wingfield".

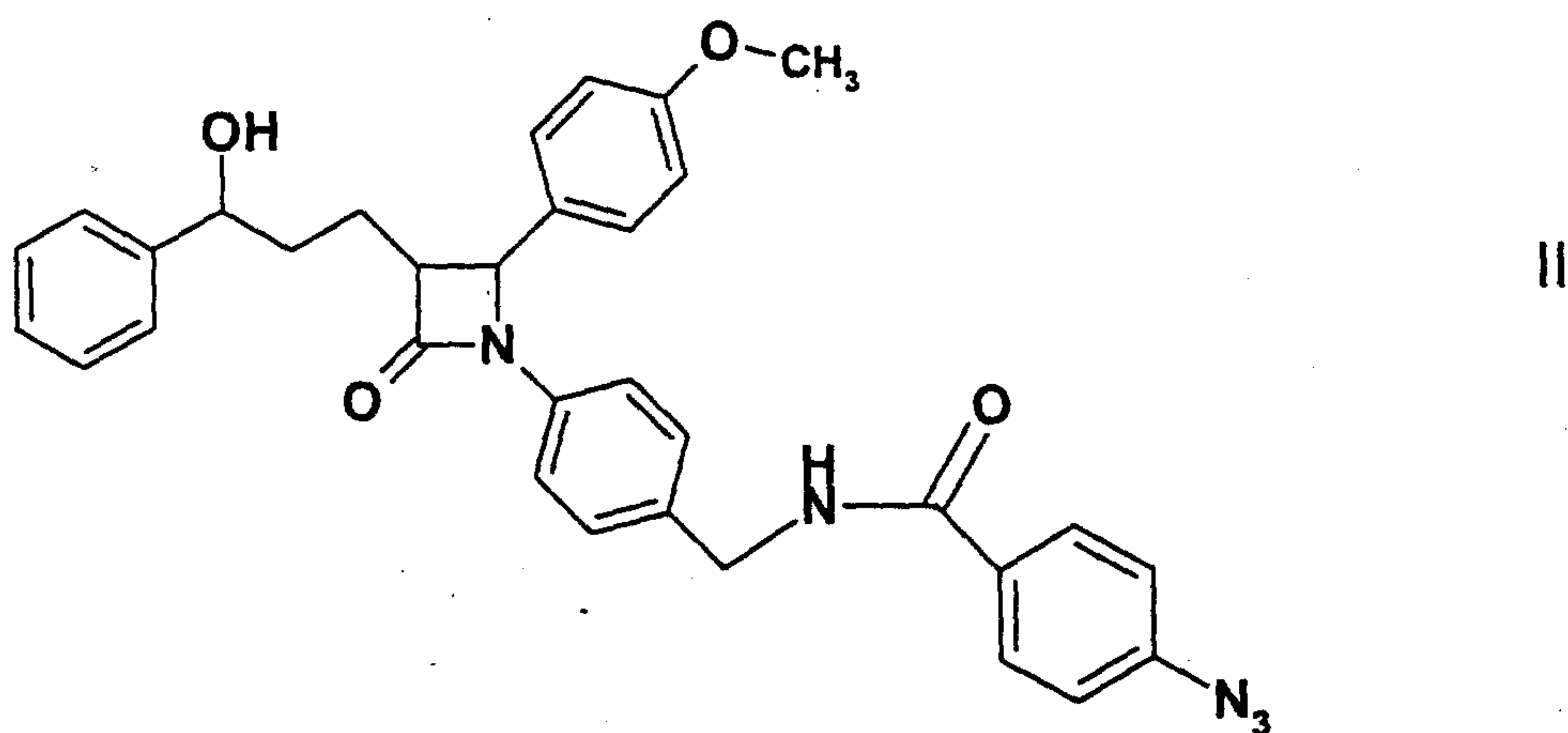
In a preferred embodiment of the invention, intestinal cells from humans, monkeys, cattle, pigs, rats, mice, rabbits or hamsters are used.

The compound preferably used as one which acts as inhibitor of intestinal cholesterol absorption and which contains a photolabile group is the following compound of the formula I, which is radiolabeled.



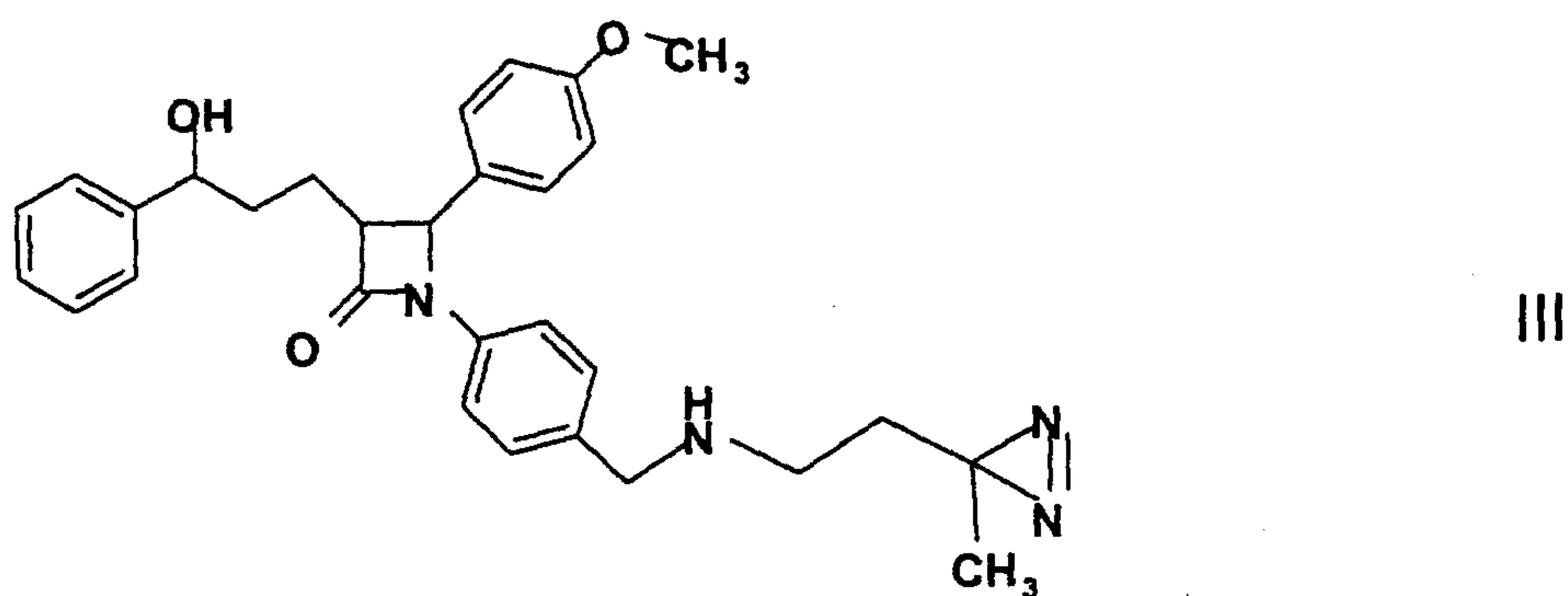
The name of the compound of the formula I is N-{4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzyl}acetamide.

In another preferred embodiment of the invention, the compound used as one which acts as inhibitor of intestinal cholesterol absorption and which contains a photolabile group is the following compound of the formula II, which is radiolabeled.



The name of the compound of the formula II is 4-azido-N-{4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzyl}benzamide.

A further compound preferably used as one which acts as inhibitor of intestinal cholesterol absorption and which contains a photolabile group is the following compound of the formula III, which is radiolabeled.



The name of the compound of the formula III is 3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)-1-(4-{[2-(3-methyl-3H-diazirin-3-yl)ethylamino]methyl}phenyl)-azetidin-2-one.

The synthesis of a compound of the formula I, II and III is described in a paragraph in the examples hereinafter.

5 The protein of the invention is able preferentially to bind an inhibitor of intestinal cholesterol absorption. The protein particularly preferably binds azetidinones or saponins. It is also preferred for the protein to be able to bind cholesterol. The inhibition of intestinal cholesterol absorption is determined, for example, using isolated human or animal intestinal cells, intestinal cell lines or parts of a human or animal intestine. This is done by determining the absorption onto said cells using
10 labeled cholesterol. The label on the cholesterol may consist of a radiolabel or another label. It is possible to use as radiolabel ^3H , ^{14}C or other isotopes which can be inserted into the molecular structure of cholesterol by synthetic methods known to the skilled worker. An inhibitor of intestinal cholesterol absorption reduces the amount of cholesterol absorbed by the intestinal cells.

15 In a preferred embodiment of the invention, the protein or parts thereof have a size of from 150 to 25 kDa. In a particularly preferred embodiment, the protein or parts thereof have a size of from 150 kDa to 32 kDa. In another particularly preferred embodiment, the protein or parts thereof have a size of from 150 kDa to 42 kDa. In a
20 very particularly preferred embodiment, the protein, in particular a part thereof, has a size of 145 kDa.

The described difference in the sizes of the protein of this invention may arise through initial formation of a precursor protein which is split up by a subsequent
25 processing into parts differing in size. Parts of the protein may be produced by enzymatic or nonenzymatic cleavage, disintegration of the protein during the experimental workup or other processes.

Embodiments of the protein of this invention have a size of 140-150 kDa, 145 kDa, 97 kDa, 90 kDa, 80 kDa, 72 kDa, 65 kDa, 63 kDa, 60 kDa, 58 kDa, 43 kDa, 41 kDa,
30 36 kDa, 33 kDa, 32 kDa or 25 kDa. In further embodiments, the protein has a size of 140-150 kDa, 145 kDa, 97 kDa, 90 kDa, 87 kDa, 80 kDa or 41 kDa. In other embodiments again the protein has a size of 140-150 kDa, 145 kDa, 97 kDa, 58 kDa, 32 kDa. In the particularly preferred embodiment, the protein has a size of

145 kDa. The size of the protein can be determined, for example, by using denaturing polyacrylamide electrophoresis comparing with size markers, by high pressure liquid chromatography comparing with size markers, gel chromatography comparing with size markers, mass spectrometry or other methods.

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A preferred embodiment of the protein is glycosylated. This glycosylated form of a protein is identified through the size of the protein decreasing when the protein is treated with a glycosidase. Details of suitable methods for checking glycosylation are to be found by the skilled worker in "Carbohydrate Biotechnology Protocols, Methods in Biotechnology, 10 (1999) Humana Press, ISBN 0-89603-563-8, Editor C. Bucke".

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The contacting of the intestinal cells or of parts of these intestinal cells, in particular of membranes, with the radiolabeled compound comprising an inhibitor of intestinal cholesterol absorption and a photolabile group can take place in conventional laboratory containers such as, for example Eppendorf vessels, centrifuged tubes or glass flasks. The underlying medium contains, for example, buffer substances, nutrient medium components, salts, trace elements and others in aqueous solution.

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A photolabile group in a molecule can be used to produce covalent linkages to a molecule, in particular a protein, located in the direct vicinity. For this purpose, the compound with the photolabile group is initially brought into the direct vicinity of the molecule to which the covalent bond is to be produced. This can take place, for example, through another part which acts as specific inhibitor - in the present case of cholesterol absorption - of a protein. Contacting of the molecules is followed by irradiation with UV light. The irradiation with UV light activates the photolabile group and initiates the production of a covalent bond with the interacting molecule, in particular with a protein. Suitable as photolabile group are, for example, diazirine, azido or carbonyl functions.

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It is possible to employ for the irradiation a conventional UV lamp like that used, for example, for visualizing polynucleotides with intercalated ethidium bromide or for sterilizing laboratory surfaces, or a photochemical reactor (obtainable inter alia from "The Southern Ultraviolet Company, Hamden, CT"). The disruption of the cells after irradiation with UV light is carried out using methods normally used for cell disruption. Examples thereof are repeated freezing and thawing, treatment of the

cells with ultrasound, the use of a French press or the addition of a detergent and enzymes. The fractionation of the proteins of the cell lysate can be carried out, for example, by precipitation with ammonium sulfate, by differential centrifugation or application of chromatographic techniques. Chromatographic techniques suitable for this purpose are, for example, denaturing or nondenaturing polyacrylamide electrophoresis in one or two dimensions, high pressure liquid chromatography, ion exchange chromatography or affinity chromatography. These techniques are familiar to the skilled worker and are dealt with in detail for example in the previously mentioned "Current Protocols in Protein Science".

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The detection of a protein after a fractionation takes place by means of the radiolabeling of the compound containing an inhibitor of intestinal cholesterol absorption and a photolabile group. A radioactive isotope which can be used for this purpose is, for example, ^3H or ^{14}C . A suitable detection method is, for example, detection of the protein which contains a covalently bonded compound by means of a film material used for X-ray photography after the protein has been introduced onto a polyacrylamide gel with the aid of polyacrylamide gel electrophoresis. Other suitable detection methods are liquid scintillation counting or flat bed scanning.

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The invention further relates to a pharmaceutical comprising a protein of the invention. This pharmaceutical can be used, for example, for the treatment of a disorder of cholesterol uptake or cholesterol excretion, of lipid disorders, atherosclerosis or cardiovascular disorders. The pharmaceutical may contain other substances or excipients which are necessary, for example, to stabilize or formulate the pharmaceutical. The contribution of the protein to the effect of the pharmaceutical can take place, for example, through binding of the cholesterol in the intestine.

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The invention also relates to the use of a protein as described above for the production of a pharmaceutical for the treatment of a disorder of cholesterol uptake or cholesterol excretion, of lipid disorders, atherosclerosis or cardiovascular disorders.

The invention further relates to a method for identifying a compound which inhibits intestinal cholesterol absorption, where the method comprises the following process steps:

- a) provision of a protein of this invention,
- 5 b) provision of a compound,
- c) contacting a protein from a) with a compound from b),
- d) determination of the binding of the compound from b) to the protein from a).

It is possible to use for the provision of a protein of the invention for carrying out a
10 method as described above in principle all cells which produce such a protein and, in particular, cells from the brush border of the intestinal tissue of mammalian organisms. It is possible to select as mammalian organisms from which these intestinal cells are obtained for example humans, monkeys, cattle, pigs, rats, mice, rabbits, hamsters or another vertebrate species. The provision can take place by
15 preparation of cell suspensions from the brush border tissue of the intestine of such organisms. Suitable intestinal material is obtained, for example, by surgical procedures. Other sources may derive from the parts of animals remaining after slaughtering. Cells of an intestinal cell line are equally suitable. To prepare suitable cell preparations, the intestinal tissue may be subjected to an enzyme treatment to
20 release single cells and then undergo differential centrifugation. The resulting cells or organelles are subsequently taken up in suitable aqueous media. These aqueous media may contain buffer substances, salts, proteins and, in addition, excipients.

The provision of a protein for carrying out the method can also take place using an in
25 vitro system. For this purpose, the protein of this invention can be obtained from cells, for example of the brush border of the intestine of mammalian organisms, with the aid of chromatographic techniques. The provision of a compound for the aforementioned method takes place, for example, by chemical synthesis. The compound may be part of a collection of chemical compounds like those resulting
30 from storage and cataloging of the chemical compounds from completed synthesis programs (called "compound libraries"). The compound may in other cases have been produced by a microorganism, in particular a bacterium, a fungus or an animal or plant species (natural substances). In the case of a natural substance, the provision can also take place by isolation from the appropriate organisms. The

contacting of a protein with a compound to carry out the method frequently takes place in aqueous solutions to which a certain proportion of a solvent such as, for example, dimethyl sulfoxide or ethanol can be admixed. The aqueous solutions usually also contain buffer substances, ions or stabilizing additions such as proteins, glycerol or others. Particular constant conditions, for example for the temperature, the pH, the ionic conditions, the concentration of the protein or of the compound, or the volume, may be advantageous for the contacting. Thus, for example, it may be beneficial to keep the temperature constant at 37°C or at room temperature during the contacting. The determination of the binding of the compound to the protein after carrying out the contacting takes place, for example, by interaction with cholesterol which is radiolabeled or labeled in another way, using the displacement of the cholesterol as a measure of the affinity of the compound for the protein.

The invention also relates to a pharmaceutical which comprises a compound which it has been possible to identify as intestinal cholesterol inhibitor using a method for identifying a compound which inhibits intestinal cholesterol absorption. The invention further relates to the use of a compound which has been identified by the method of the invention for producing a pharmaceutical for the treatment of a disorder of cholesterol uptake or cholesterol excretion, of lipid disorders, atherosclerosis or cardiovascular disorders.

The invention also relates to a method for obtaining a protein of the invention, where the method comprises the following process steps:

- a) provision of a cell comprising a protein of the invention,
- b) provision of a chemical conjugate comprising a polymer, where the polymer is possibly covalently linked via a spacer to an inhibitor of intestinal cholesterol absorption,
- c) disruption of the cell from a)
- d) contacting the disrupted material from c) with a chemical conjugate from b),
- e) separation of the unbound proteins and other molecules of the disrupted material from c) from the chemical conjugate,
- f) elution of the proteins which have entered into a stable connection with chemical conjugate after contacting as in d) and, where appropriate, further purification.

A vertebrate intestinal cell can be used as cell containing a protein of this invention. It is possible to select as mammalian organisms from which such intestinal cells are obtained, for example humans, monkeys, cattle, pigs, rats, mice, rabbits or hamsters. The provision can take place by preparation of cell suspensions from the brush border tissue of the intestine. Suitable intestinal material is obtained, for example, by surgical procedures. Other sources may be parts of animals remaining behind after slaughtering. Cells of an intestinal cell line are also suitable. To release single cells, the intestinal tissues, for example, are subjected to an enzyme treatment or a differential centrifugation. The resulting preparations of the cells or organelles are then taken up in suitable aqueous media. These aqueous media may contain buffer substances, salts, proteins and, in addition, excipients. The cell preparation is subjected to a disruption in order to obtain a protein of this invention. The disruption of the cells is carried out using the methods normally used for cell disruption. Examples thereof are repeated freezing and thawing, treatment of the cells with ultrasound, the use of a French press or the addition of a detergent and enzymes.

The provision of a chemical conjugate consisting of a polymer, where the polymer is possibly covalently linked via a spacer to an inhibitor of intestinal cholesterol absorption, takes place by chemical synthesis. After the synthesis, the conjugate is taken up in an aqueous or organic solvent. Stable connection of a protein to this chemical conjugate can take place by affinity binding, via a hydrophilic or hydrophobic interaction or in another way. This connection of the protein to the chemical conjugate can be dissolved again by introducing suitable eluents. Such suitable eluents contain, for example, high concentrations of a substance which behaves as competitor with an affinity or other interaction.

The disrupted cells which have been produced as just described are contacted with the chemical conjugate. For this purpose, the chemical conjugate can be introduced as suspension into a chromatography column beforehand. After packing of the column, the disrupted material is then loaded onto the affinity chromatography material and eluted with an aqueous solvent which may contain buffer substances, salts, proteins and excipients, in particular stabilizers, solubilizers or preservatives. The elution separates the unbound proteins and other molecules which do not correspond to a protein of this invention from the chemical conjugate. The proteins

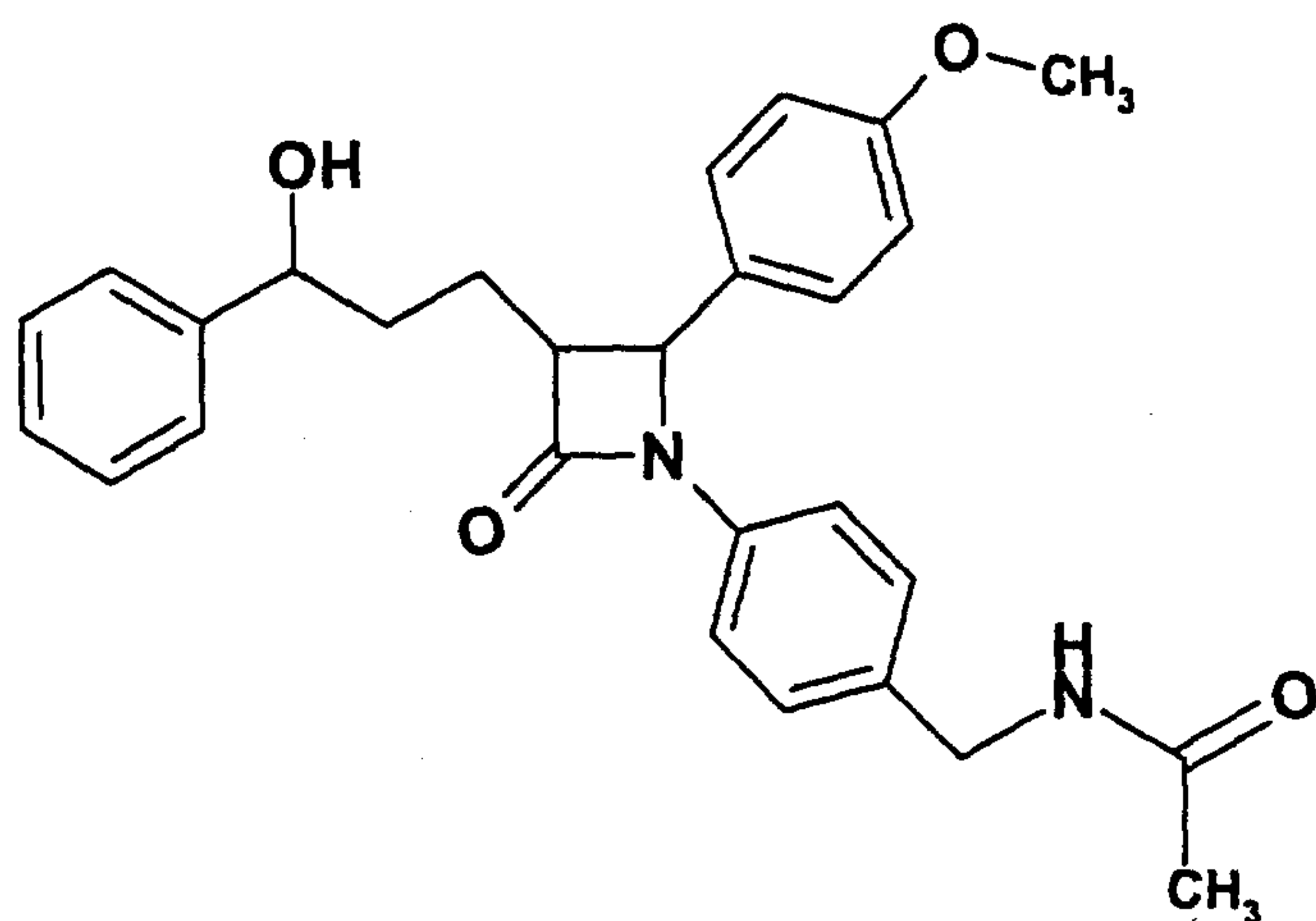
which have entered into a stable connection with the chemical conjugate after contacting with the disrupted material are then eluted. This can take place, for example, by using increasing concentrations of the inhibitor which is present in the chemical conjugate. An alternative possibility is to use another inhibitor of intestinal cholesterol absorption. The eluted proteins can be subjected to another purification, for example by other chromatographic techniques such as ion exchange chromatography, polyacrylamide electrophoresis, gel chromatography, high pressure liquid chromatography.

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- 10 In a preferred method for obtaining a protein of the invention as described above, the chemical conjugate comprises a polymer, where appropriate a linker, and an inhibitor of cholesterol absorption from the saponin or 2-azetidinone series, in particular a compound of the formula I, II or III of this invention.
- 15 The invention further relates to a chemical conjugate comprising a polymer, where the polymer is possibly covalently linked via a spacer to an inhibitor of intestinal cholesterol absorption. Such an inhibitor is, in a preferred embodiment, a saponin or a compound of the formula I, II or III.
- 20 The invention further relates to a chemical conjugate of this invention, this conjugate comprising a bound protein of this invention.

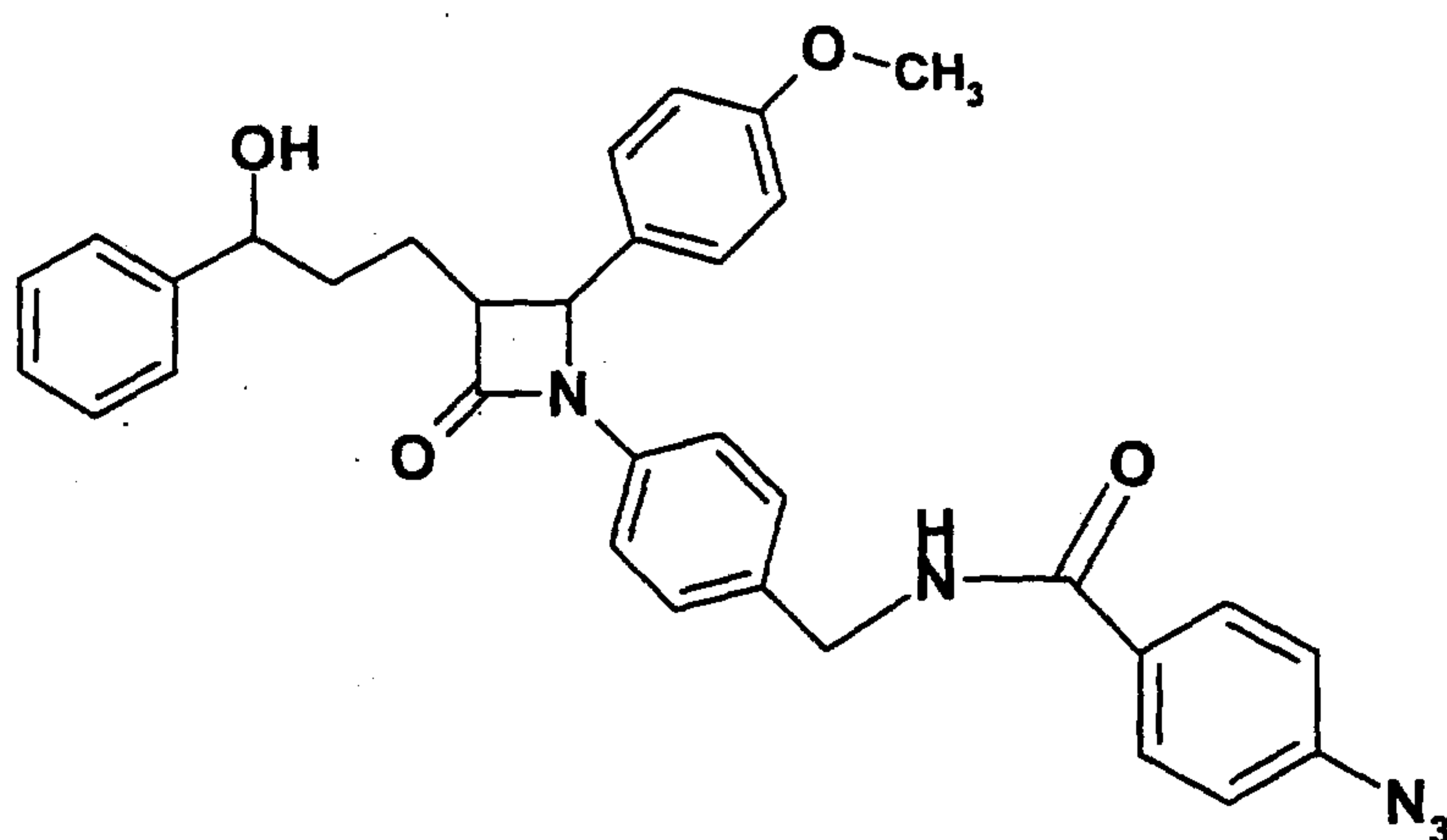
The preparation of a chemical conjugate by chemical synthesis is described in the examples.

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The invention relates to a compound of the formula I.

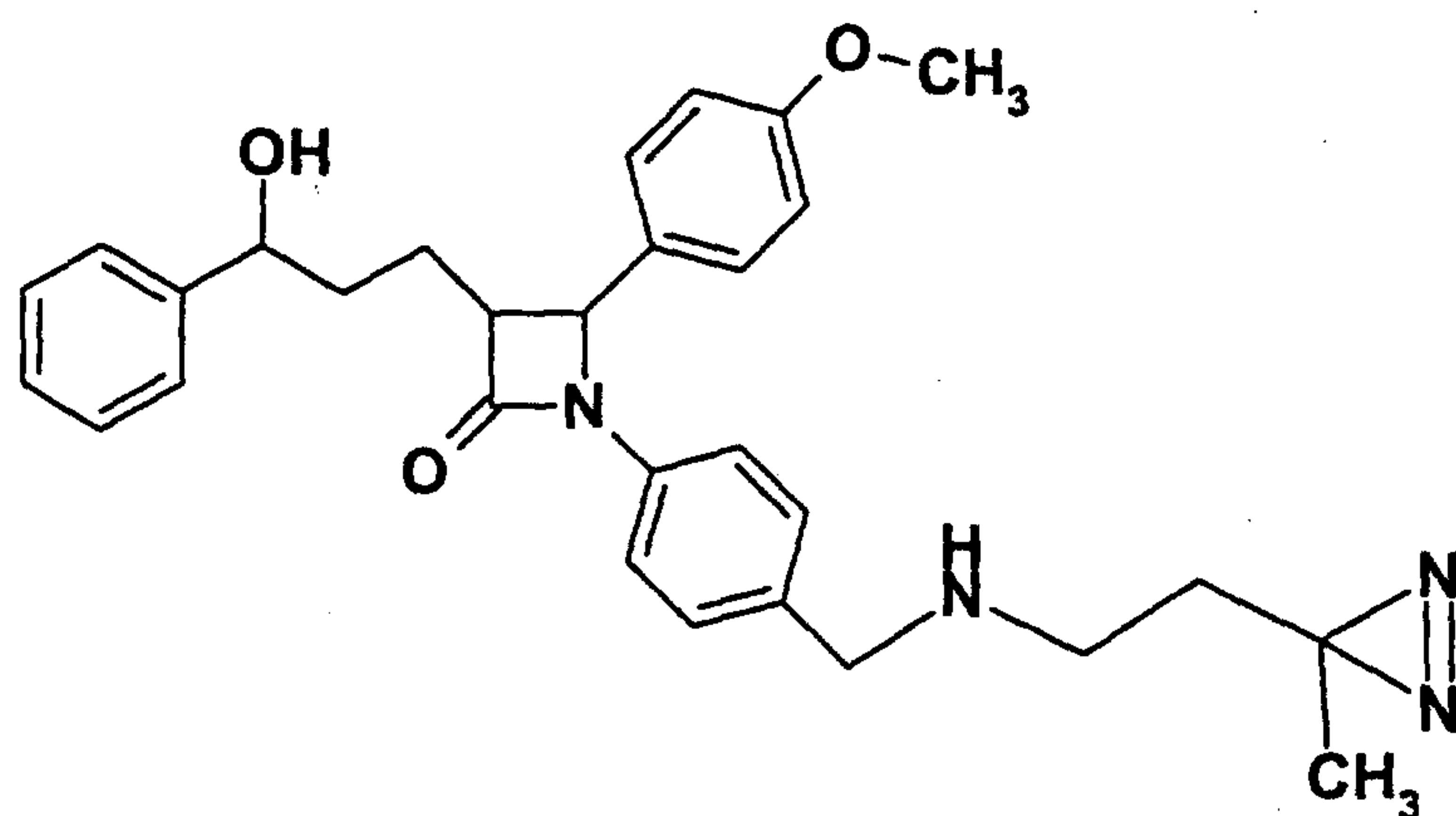


The invention further relates to a compound of the formula II.



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The invention also relates to a compound of the formula III.



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- 10 A compound of the formula I, II or III can be used to detect a protein of this invention (photolabel). A compound of the formula I, II or III is also suitable as inhibitor of a cholesterol-absorbing protein. The present invention therefore also relates to a

diagnostic kit which comprises at least one compound of the formula I, II or III, and reagents for carrying out an assay for detecting a protein of this invention.

The invention also relates to the compounds 4-[3-(3-bromo-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxo-azetidin-1-yl]benzonitrile, 4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzonitrile or 1-(4-aminomethylphenyl)-3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)azetidin-2-one. These compounds which have just been mentioned can be used to prepare a compound of the formula I, II or III.

Examples:

Inhibitors of intestinal cholesterol absorption were synthesized (compounds of the formula I, II or III). These compounds contain photolabile groups which can be crosslinked with other molecules, in particular proteins, by means of UV light, and are radiolabeled with a specific activity greater than 1 Ci/mmol. These compounds are used to identify a protein of intestinal cholesterol absorption.

Example 1:

Photoaffinity and binding studies:

Vesicles from the brush border tissue of the small intestine of rabbits were isolated by methods known to the skilled worker (Kramer et al. J. Biol. Chem. 268, 18035 – 18046 (1993)). The photoaffinity labeling using a radiolabeled compound of the formula I, II or III of this invention was carried out in a photochemical reactor of the Rayonet RPR-100 type (obtainable from "The Southern Ultraviolet Company, Hamden, CT"). The brush border vesicles thereof (100 to 200 µg of protein) were incubated with one of the compounds in a volume of 200 µl in 10 mM Tris/Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol at 20°C in the dark for 5 min. In place of the brush border vesicles it is also possible to use organelles, in particular membranes thereof. The statements hereinafter apply correspondingly to these. The incubation in the dark was followed by irradiation with UV light of 254 nm for

20 seconds or 60 seconds. The brush border vesicles were then washed twice with the buffer mentioned. The proteins were precipitated by conventional techniques such as, for example, addition of ethanol, addition of a salt or detergent, heating, repeated freezing and thawing, or another suitable method known to the skilled worker, and fractionated by SDS polyacrylamide electrophoresis. The radiolabeled proteins were detectable by LSC or fluorography. The affinity of the labeled proteins from the brush border tissue for the compounds was in the range from 1 to 10 nM.

Identification of an intestinal protein which absorbs cholesterol.

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Vesicles were prepared from brush border membranes of the rabbit intestine. They were labeled by means of a radiolabeled compound of the formula I, II or III. The proteins were precipitated and fractionated on an SDS polyacrylamide gel.

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On use of the compound of the formula I it was possible to label proteins with a molecular weight of 145 kDa, 97 kDa, 72 kDa, 63 kDa, 58 kDa, 41 kDa 32 kDa and 25 kDa (Fig. 1a). With the compound of the formula II it was possible to label compounds with a molecular weight of 145 kDa, 97 kDa, 87 kDa and 41 kDa. The greatest incorporation was shown by the protein with the molecular weight of

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145 kDa. It was possible to show by solubilization experiments with nonionic or zwitterionic detergents and sodium carbonate that the proteins with the molecular weight of 145 kDa, 97 kDa, 58 kDa and 32 kDa are integral membrane proteins. The molecular weights of the proteins are stated subject to a certain range of uncertainty which is caused by the SDS polyacrylamide gel electrophoresis method used, but is also known for other corresponding methods. The variations in the molecular weights are in the region of up to $\pm 10\%$. The stated values represent the means of a plurality of experiments. In the case of the protein with the stated molecular weight of 145 kDa, the determinations of the molecular weight in 10 experiments carried out independently of one another by SDS polyacrylamide gel electrophoresis resulted in

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Further photolabeling studies were carried out with known inhibitors of intestinal cholesterol absorption. Derivatives of S3302 were prepared for this purpose. The compound S3302 is a racemate of the cholesterol inhibitor SCH 48461. This

compound very efficiently inhibits intestinal cholesterol absorption in rabbits, hamsters, dogs, rhesus monkeys and humans (Davis et al., Atherosclerosis 109, 162 – 163 (1994); Bergman et al.; XII International Symposium on Drugs affecting Lipid Metabolism; conference reports (1995)). The compound SCH 58235 (references just
5 mentioned) shows in hamsters an effect which is increased 50-fold on direct comparison. This effect is explained by the introduction of an additional (3S)-hydroxyl group in position R2. It was possible to prepare an analogous hydrogen derivative of SCH 48461 (= S6503) which showed similar activity to SCH 58235 in an in vivo experiment. S6503 brings about, in analogy to its strong in vivo
10 effect on cholesterol absorption, a greater inhibition, compared with S3302, of the labeling of the protein with the molecular weight of 145 kDa. None of the inhibitors of cholesterol absorption used showed a noteworthy effect on bile salts, glucose, oligopeptides, alanine, fatty acids or the Na⁺/bile salt transport route in the in vivo experiments.

15 Photoaffinity studies with brush border membranes isolated from rabbits given a diet with large amounts of cholesterol for 10 days showed distinctly greater labeling of the protein with the molecular weight of 145 kDa (Fig. 2).

20 It is evident from the above statements that it was possible to prepare a protein which brings about intestinal cholesterol absorption. It was thus possible to achieve the object of this invention.

Example 2:

25 Provision of a chemical conjugate of a polymer and a compound which acts as inhibitor of intestinal cholesterol absorption

An aminoalkyl derivative of a compound which acts as inhibitor of intestinal cholesterol absorption (5-100 µmol), in particular a compound of the formula I, II or III
30 of this invention, is dissolved in 1-5 ml buffer/0.1 M NaHCO₃/0.5 M NaCl (pH 8.3). Polymeric support materials containing activated carboxyl groups with or without spacer, such as, for example, a Hi-TrapTM column with an N-hydroxysuccinimidyl-activated hexylcarboxylic acid as spacer (Pharmacia Biotechnology) are prepared for conjugation in accordance with the manufacturer's instructions. The activated matrix

is incubated with the above solution of an aminoalkyl derivative of a cholesterol absorption inhibitor for 0.5-5 h. Unreacted activated carboxyl groups are then neutralized by sequential washing several times with in each case 5-15 ml of the following buffers:

- 5 0.5 M ethanolamine/0.5 M NaCl (pH 8.3)
 0.1 M sodium acetate/0.5 M NaCl (pH 4.0)

The conjugate column is then prepared for the affinity chromatography by washing with 10 mM sodium phosphate buffer (pH 7.4)/1% n-octyl glucoside.

10 Concentration of binding proteins for cholesterol absorption inhibitors by affinity chromatography

Biological membranes from cells which contain cholesterol transport proteins (2-20 mg of protein), such as, for example, brush border membranes from intestinal
15 cells, are solubilized at a protein concentration of 0.5-2.5 mg/ml in 10 mM sodium phosphate buffer (pH 7.4)/1% n-octyl glucoside at 4°C for 1 h. After removal of remaining particulate material by centrifugation, the clear supernatant solution, which contains the solubilized membrane proteins, is loaded onto the conjugate column described above, and the elution of protein is followed by the UV absorption. After
20 unretarded proteins have been washed out, the specific binding proteins for cholesterol absorption inhibitors which are adhering to the column are eluted by applying solutions which contain cholesterol absorption inhibitors in concentrations of 1-10 mM or which contain 1-5% of nonionic detergents such as Triton X-100™. The binding proteins for cholesterol absorption inhibitors in these eluates are then
25 separated by electrophoretic fractionation and their molecular size is determined. Their amino acid sequence can be determined by sequencing by methods familiar to the skilled worker by cutting out the appropriate protein bands, or antibodies against the individual proteins can be raised by subcutaneous deposition of the pieces of gel which contain the fractionated proteins in various animal species.

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Example 3:

The preparation of a compound of the formula I, II or III and of a radiolabeled derivative of a compound of the formula I, II or III is described below:

4-[3-(3-Bromo-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzonitrile

16.0 g of 4-[2-(4-methoxyphenyl)-4-oxo-3-(3-phenylpropyl)azetidin-1-yl]benzonitrile are dissolved in 180 ml of tetrachloromethane, and 9.4 g of N-bromosuccinimide and 940 mg of benzoyl peroxide are added. The mixture is heated to reflux with stirring and monitored by thin-layer chromatography. The reaction is complete after about 30-50 min. The solution is mixed at room temperature with ethyl acetate and then washed with saturated sodium bisulfite solution and water, dried over sodium sulfate and concentrated in vacuo. The residue is purified on silica gel with ethyl acetate/heptane = 1:2 as mobile phase. The bromide is obtained as a viscous oil. MS(FAB): 477 ($M+H^+$), 475 (M_2+H^+) [$C_{26}H_{23}BrN_2O_2$ $M=475$].

4-[3-(3-Hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzonitrile

17 g of 4-[3-(3-bromo-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzonitrile are dissolved in 500 ml of dioxane and, after addition of 34 ml of ~40 percent tetrabutylammonium trifluoroacetate solution, heated to reflux for 24 h. The mixture was concentrated to about half the volume, mixed with water and methyl tert-butyl ether and separated into the phases. The organic phase is concentrated, and the residue is stirred with 500 ml of ethanol and 200 ml of concentrated ammonia at room temp. for 1 h. It is then again concentrated in vacuo, and the crude product is purified on silica gel with dichloromethane/methanol = 30:1 as mobile phase. The alcohol is obtained as an oil. MS (ESI): 413 ($M+H^+$), 395 ($M+H^+-H_2O$) [$C_{26}H_{24}N_2O_3$ $M=412$].

1-(4-Aminomethylphenyl)-3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)-azetidin-2-one

7 g of 4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzonitrile are dissolved in 200 ml of ethanol and, after addition of 15 ml of concentrated ammonia, hydrogenated with 7 g of 50% aqueous Raney nickel under 50 bar of hydrogen. The crude product obtained after removal of the catalyst by

filtration with suction and removal of the solvent in vacuo is purified on silica gel with dichloromethane/methanol/ammonia = 200:10:1. The amine is obtained. MS (FAB, + LiCl): 423 (M+Li⁺) [C₂₆H₂₈N₂O₃ M=416].

5

4-Azido-N-{4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxo-azetidin-1-yl]benzyl}benzamide

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140 mg of 1-(4-aminomethylphenyl)-3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)azetidin-2-one are dissolved in 5 ml of dioxane, and 0.1 ml of tributylamine is added. To this mixture are added, under subdued light, 88 mg of 4-azidobenzoic acid succinimide ester, followed by stirring at room temperature for about 1 h. The crude product remaining after concentration is purified on silica gel with ethyl acetate/heptane = 1:1 as mobile phase. MS (FAB): 562 (M+H⁺), 544 (M+H⁺-H₂O)

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[C₃₃H₃₁N₅O₄ M=561].

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The radiolabeled benzamide is obtained analogously on a small scale in high dilution using tritium- or ¹⁴C-labeled azidobenzoic acid activated ester (from Amersham) and coelutes in a thin-layer chromatogram with unlabeled 4-azido-N-{4-[3-(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzyl}benzamide.

N-{4-[3-(3-Hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-benzyl}-acetamide

25

100 mg of 1-(4-aminomethylphenyl)-3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)azetidin-2-one are dissolved in 20 ml of methanol and, 0.3 ml of pyridine followed by 0.3 ml of acetic anhydride are added. The reaction is complete after 30 min at room temperature and, after concentration in vacuo, purification is carried out on silica gel with ethyl acetate as mobile phase. MS (ESI): 459(M+H⁺), 441

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(M+H⁺-H₂O) [C₂₈H₃₀N₂O₄ M=458].

The radiolabeled acetamide is obtained analogously on a small scale in high dilution using tritium- or ¹⁴C-labeled acetic anhydride and coelutes with unlabeled N-{4-[3-

(3-hydroxy-3-phenylpropyl)-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]benzyl}acetamide in a thin-layer chromatogram.

3-(3-Hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)-1-(4-{{2-(3-methyl-3H-diazirin-3-yl)ethylamino}methyl}phenyl)azetidin-2-one

100 mg of 1-(4-aminomethylphenyl)-3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)azetidin-2-one are dissolved in 5 ml of dimethylformamide and stirred with 2-(3-methyl-3H-diazirin-3-yl)ethyl toluene-4-sulfonate under subdued light at 60-70°C for 6 h. Water is added and, after extraction with ethyl acetate, the solvent is removed in vacuo. Then dry toluene is added and the mixture is concentrated in vacuo twice. The crude product is purified on silica gel using dichloromethane/methanol = 20:1 as mobile phase.

MS (ESI): 499 (M+H⁺) [C₃₀H₃₄N₄O₃ M=498].

The radiolabeled azetidinone is obtained in the same way using tritium- or ¹⁴C-labeled diazirine and coelutes in a thin-layer chromatogram with unlabeled 3-(3-hydroxy-3-phenylpropyl)-4-(4-methoxyphenyl)-1-(4-{{2-(3-methyl-3H-diazirin-3-yl)ethylamino}methyl}phenyl)azetidin-2-one.

Explanation of the figures:

Fig 1a, Fig. 1b:

Labeling of proteins of rabbit brush border membrane with radiolabeled compounds of the formula I or II. The proteins were precipitated and fractionated on an SDS polyacrylamide gel. The amount of radioactivity was determined by quantitative fluorography. The distance from the application point in cm is plotted on the horizontal axis of the diagram, and the result of counting in ³H dpm is plotted on the vertical axis. Fig. 1a shows in the upper part the distribution of the radioactivity after labeling with radiolabeled compound of the formula II and fluorographic detection.

A compound of the formula I was used for labeling in Fig. 1a, and a compound of the formula II was used in Fig. 1b.

Specifically labeled proteins are evident from the peaks.

Fig 2a, 2b:

Rabbits were fed for 10 days with a diet containing high concentrations of cholesterol. Then vesicles from the brush border membranes of these animals were prepared and subsequently treated with a compound of the formula I or II as described, and then the proteins were precipitated and fractionated on an SDS polyacrylamide gel. The amount of radioactivity was determined by quantitative fluorography. The distance from the application point in cm is plotted on the horizontal axis of the diagram, and the result of counting in ^3H dpm is plotted on the vertical axis. A compound of the formula I was used for labeling in Fig. 2a, and a compound of the formula II was used in Fig. 2b.

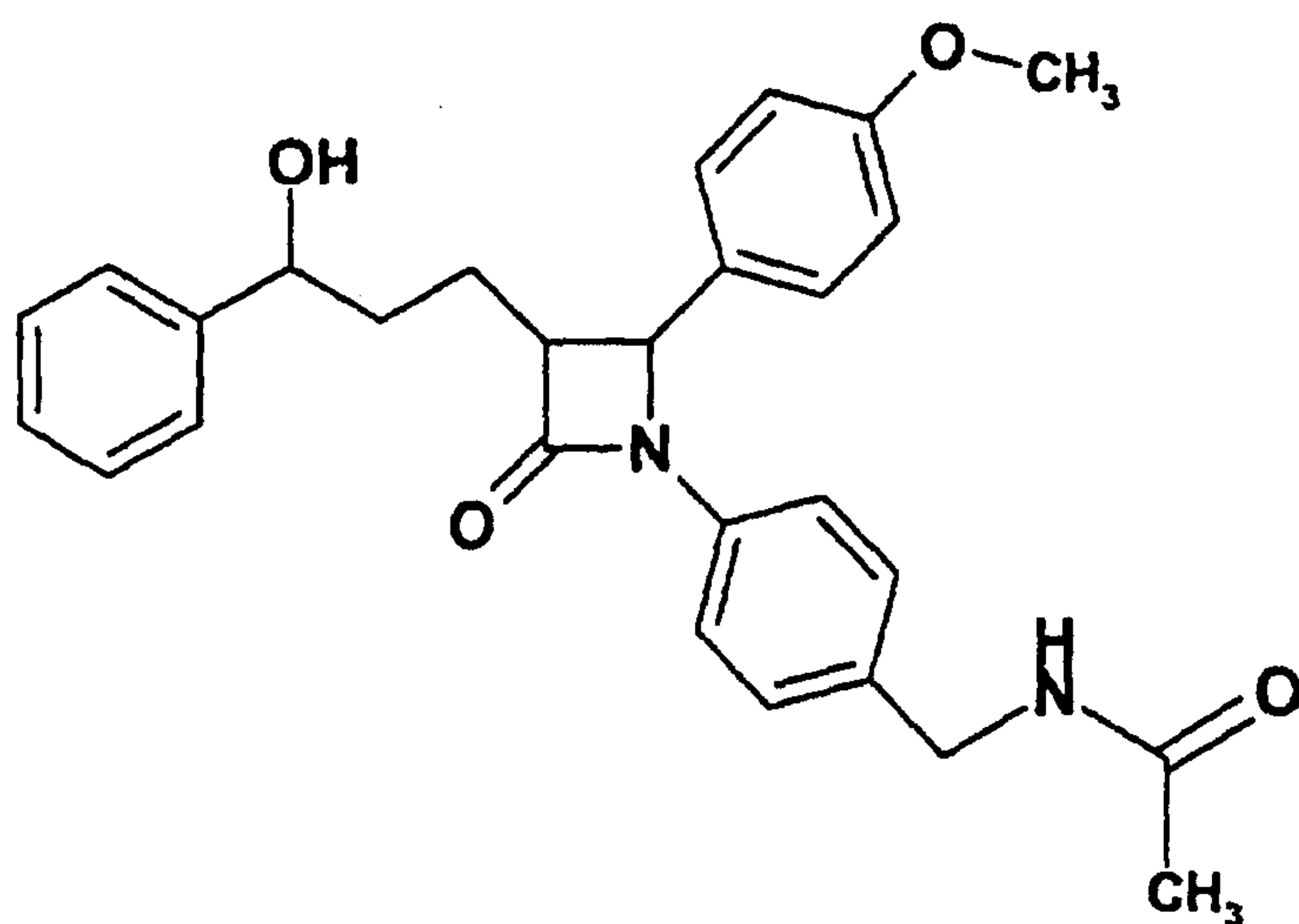
A specific labeling of a protein with a molecular weight of 145 kDa was obtained.

Abbreviations:

h	hour(s)
15 LSC	liquid scintillation counting
MS (FAB)	mass spectrum (fast atom bombardment)
MS (ESI)	mass spectrum (electrospray ionization)
M	mass

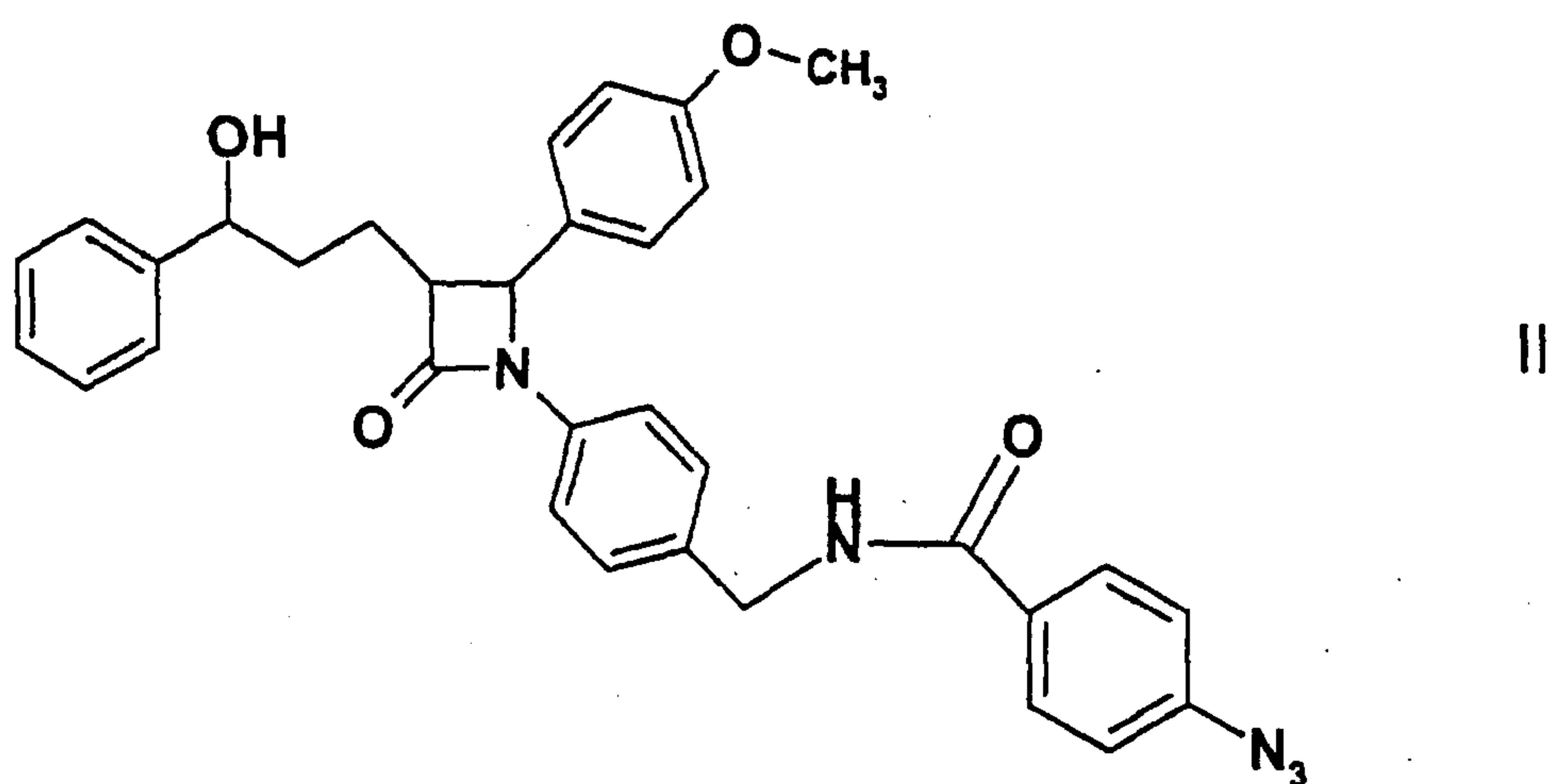
Claims

1. A cholesterol-absorbing protein from the intestinal tissue of a mammalian organism which contains a covalently bound radiolabeled compound which acts as an inhibitor of intestinal cholesterol absorption and contains a photolabile group, obtainable by the following process steps:
- 5 acts as an inhibitor of intestinal cholesterol absorption and contains a photolabile group, obtainable by the following process steps:
- a) provision of intestinal cells or parts of these intestinal cells of a mammalian organism,
- b) provision of the radiolabeled compound which acts as inhibitor of intestinal cholesterol absorption and contains a photolabile group,
- 10 c) contacting the intestinal cells or parts of these intestinal cells from a) with the compound from b),
- d) irradiation of the mixture from c) with UV light,
- e) disruption of the cells after irradiation as in d),
- 15 f) fractionation of the components of the disrupted cells after disruption as in e) by ammonium sulfate precipitation, differential centrifugation or chromatographic techniques,
- g) detection, after fractionation as in f), of a protein which contains the covalently bound compound from b),
- 20 wherein the protein has a size of 145 kDa and is glycosylated, wherein the compound in step b) is a compound having the following formula I



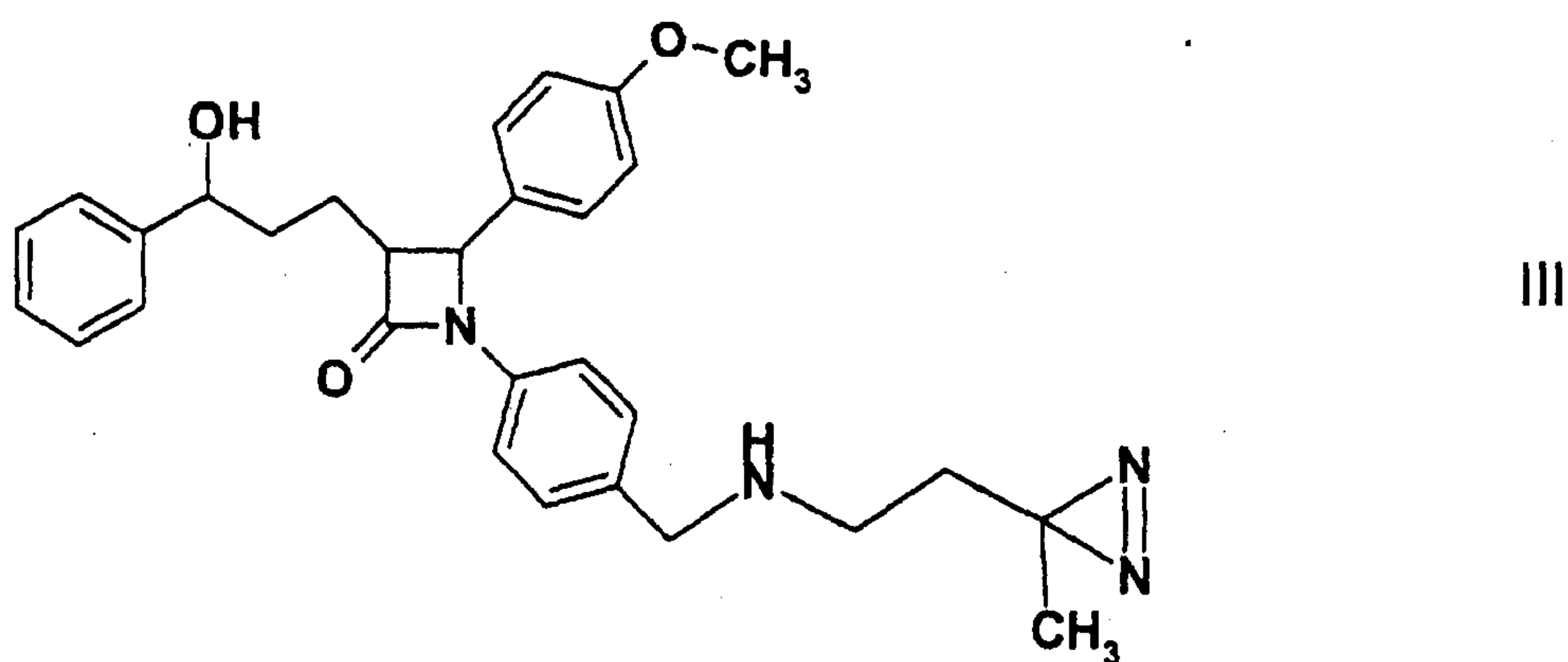
or

- 5 wherein the compound in step b) is a compound having the following formula
II



10 or

- wherein the compound in step b) is a compound having the following formula
III



15

2. The protein as claimed in claim 1, where the intestinal cells in step a) originate from humans, monkeys, rats, cattle, pigs, mice, rabbits or hamsters.
3. The protein as claimed in claim 1 or 2, wherein the protein is able to bind
5 an inhibitor of intestinal cholesterol absorption.
4. The protein as claimed in claim 3, wherein the protein is able to bind azetidinones or saponins.
- 10 5. The protein as claimed in any one of claims 1 to 4, wherein the protein is able to bind cholesterol.
6. A pharmaceutical comprising the protein as claimed in any one of claims 1 to 5 and a pharmaceutically acceptable excipient.
15
7. A use of the protein as claimed in any one of claims 1 to 5 for the production of a pharmaceutical for the treatment of a disorder of cholesterol uptake or cholesterol excretion, of a lipid disorder, atherosclerosis or cardiovascular disorder.
20
8. A use of the protein as claimed in any one of claims 1 to 5 for treating a disorder of cholesterol uptake or cholesterol excretion, of a lipid disorder, atherosclerosis or cardiovascular disorder.
- 25 9. An *in vitro* method for identifying a compound which inhibits intestinal cholesterol absorption, wherein the method comprises the following process steps:
 - a) provision of the protein as claimed in any one of claims 1 to 5,
 - b) provision of a compound,
 - 30 c) contacting the protein from a) with the compound from b),
 - d) determination of the binding of the compound from b) to the protein from a) by determining the displacement of cholesterol which interacts with the protein from a), wherein displacement of cholesterol indicates that the compound from b) inhibits intestinal cholesterol absorption.

10. The *in vitro* method of claim 9, wherein the displacement is by the compound from b).

5 11. A method for obtaining a cholesterol-absorbing protein, wherein the method comprises the following process steps:

a) provision of intestinal cells or parts thereof of a mammalian organism,

b) provision of a chemical conjugate comprising a polymer, where the polymer is covalently linked via a spacer to an inhibitor of intestinal cholesterol
10 absorption, wherein said inhibitor is a compound of the formula I, II or III as defined in claim 1,

c) disruption of the cell from a),

d) contacting the disrupted cell from c) with the chemical conjugate from b),

e) separation of the unbound proteins and other molecules of the disrupted
15 material from c) from the chemical conjugate,

f) elution of the proteins which have entered into a stable connection with the chemical conjugate after contacting as in d) and, where appropriate, further purification;

wherein the protein has a size of 140 to 150 kDa.

20

12. The method as claimed in claim 11, where the intestinal cells in step a) originate from humans, monkeys, rats, cattle, pigs, mice, rabbits or hamsters.

13. The method as claimed in claim 11 or 12, wherein the protein is able to
25 bind an inhibitor of intestinal cholesterol absorption.

14. The method as claimed in claim 13, wherein the protein is able to bind azetidinones or saponins.

30 15. The method as claimed in any one of claims 11 to 14, wherein the protein is able to bind cholesterol.

16. The method as claimed in any one of claims 11 to 15, wherein the protein has a size of 145 kDa.

17. The method as claimed in any one of claims 11 to 16, wherein the protein is glycosylated.

