Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2917767 C 2022/05/03

(11)(21) 2 917 767

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2014/07/14
(87) Date publication PCT/PCT Publication Date: 2015/01/15
(45) Date de délivrance/Issue Date: 2022/05/03
(85) Entrée phase nationale/National Entry: 2016/01/08
(86) N° demande PCT/PCT Application No.: EP 2014/001925
(87) N° publication PCT/PCT Publication No.: 2015/003816
(30) Priorité/Priority: 2013/07/12 (EP13003539.7)

(51) Cl.Int./Int.Cl. *C12N 15/52* (2006.01), *C07C 237/44* (2006.01), *C12P 13/02* (2006.01)

(72) Inventeurs/Inventors:
BAUMANN, SASCHA, DE;
HERRMANN, JENNIFER, DE;
MOHR, KATHRIN, DE;
STEINMETZ, HEINRICH, DE;
GERTH, KLAUS, DE;
RAJU, RITESH, DE;
...

(73) Propriétaire/Owner:
HELMHOLTZ-ZENTRUM FUR INFEKTIONSFORSCHUNG GMBH, DE

(74) Agent: FASKEN MARTINEAU DUMOULIN LLP

(54) Titre : CYSTOBACTAMIDES
(54) Title: CYSTOBACTAMIDES

(57) Abrégé/Abstract:
The present invention provides cystobactamides of formula (I): R^1-Ar^1-L^1-Ar^2-L^2-Ar^3-L^3-Ar^4-L^4-Ar^5-R^2 and the use thereof for the treatment or prophylaxis of bacterial infections.

CA 2917767 C 2022/05/03
(11)(21) 2 917 767
(13) C

(72) **Inventeurs(suite)/Inventors(continued)**: MULLER, ROLF, DE; HAMED, MOSTAFA, DE; WANG, LIANG LIANG, DE; HARTMANN, ROLF, DE; ELGAHER, WALD A.M., DE; KIRSCHNING, ANDREAS, DE; MORENO, MARIA, DE; GILLE, FRANZISKA, DE; HUETTEL, STEPHAN, DE

Innovation, Sciences et Développement économique Canada
Office de la propriété intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

Bureau canadien des brevets *Canadian Patent Office*

Certificat de correction

Certificate of Correction

Canadian Patent No. 2,917,767
Granted: 3 May 2022 (03-05-2022)

Les corrections suivantes sont faites en raison de l'article 107 des *Règles sur les brevets* et le brevet doit être lu tel que corrigé.

The following corrections are made pursuant to section 107 of the *Patent Rules* and the patent should read as corrected.

In the Patent Grant:

The inventor ELGAHER, WALD A.M.
should be read as ELGAHER,
WALID A. M.

2 June 2022 (02-06-2022)

Date

Canada

(CIPO 25)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 January 2015 (15.01.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/003816 A3

(51) **International Patent Classification**:
C12N 15/52 (2006.01) *C07C 237/44* (2006.01)
C12P 13/02 (2006.01)

(21) **International Application Number**:
PCT/EP2014/001925

(22) **International Filing Date**:
14 July 2014 (14.07.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
13003539.7 12 July 2013 (12.07.2013) EP

(71) **Applicant**: **HELMHOLTZ-ZENTRUM FÜR INFEKTIONSFORSCHUNG GMBH** [DE/DE]; Inhoffenstraße 7, 38124 Braunschweig (DE).

(72) **Inventors**: **BAUMANN, Sascha**; Gartenstr. 8, 66125 Saarbrücken (DE). **HERRMANN, Jennifer**; Hohe Wacht 63, 66119 Saarbrücken (DE). **MOHR, Kathrin**; Husarenstr. 40, 38102 Braunschweig (DE). **STEINMETZ, Heinrich**; Magdalenenweg 15, 31139 Hildesheim (DE). **GERTH, Klaus**; Am Butterbusch 17, 38124 Braunschweig (DE). **RAJU, Ritesh**; Gausstr. 81/75, 66123 Saarbrücken (DE). **MÜLLER, Rolf**; Kirkelerstr. 114, 66440 Blieskastel (DE).

(74) **Agents**: **FORSTMEYER, Dietmar** et al.; BOETERS & LIECK, Oberanger 32, 80331 München (DE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) **Date of publication of the international search report**:
12 March 2015

WO 2015/003816 A3

(54) **Title**: CYSTOBACTAMIDES

(57) **Abstract**: The present invention provides cystobactamides of formula (I): R^1-Ar^1-L^1-Ar^2-L^2-Ar^3-L^3-Ar^4-L^4-Ar^5-R^2 and the use thereof for the treatment or prophylaxis of bacterial infections.

Cystobactamides

Cystobactamides are novel natural products that have been isolated from myxobacterium *Cystobacter velatus* (MCy8071; internal name: *Cystobacter ferrugineus*). Cystobactamides exhibit a good antibiotic activity, especially against selected Gram-negative bacteria, such as *E. coli*, *P. aeruginosa*, and *A. baumannii*, as well as a broad spectrum activity against Gram-positive bacteria.

The present invention provides compounds of formula (I)

$$R^1\text{-}Ar^1\text{-}L^1\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

(I)

wherein

Ar^1 is an optionally substituted phenylene group or an optionally substituted heteroarylene group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;

Ar^2 is an optionally substituted phenylene group or an optionally substituted heteroarylene group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;

Ar^3 is an optionally substituted phenylene group or an optionally substituted heteroarylene group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;

Ar^4 is absent or an optionally substituted phenylene group or an optionally substituted heteroarylene group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;

Ar^5 is absent or an optionally substituted phenylene group or an optionally substituted heteroarylene group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;

L^1 is a bond, an oxygen atom, a sulphur atom or a group of formula NH, CONH, NHCO, COO, OCO, $CONR^3$, NR^3CO, OCONH, NHCOO, NHCONH, $OCONR^3$, NR^3COO, NR^3CONR^4, NR^3, $-CNR^3-$, -CO-, -SO-, $-SO_2-$, $-SO_2NH-$, $-NHSO_2-$, $-SO_2NR^3-$, $-NR^3SO_2-$, $-COCH_2-$, $-CH_2CO-$, $-COCR^3R^4-$, $-CR^3R^4CO-$, -NHCSNH-, $-NR^3CSNR^4$, -CH=CH-, $-CR^3=CR^4-$, or a heteroarylene group having 5 or 6 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, or a heteroalkylene group;

L^2 is a bond, an oxygen atom, a sulphur atom or a group of formula NH, CONH, NHCO, COO, OCO, $CONR^3$, NR^3CO, OCONH, NHCOO, NHCONH, $OCONR^3$, NR^3COO, NR^3CONR^4, NR^3, $-CNR^3-$, -CO-, -SO-, $-SO_2-$, $-SO_2NH-$, $-NHSO_2-$, $-SO_2NR^3-$, $-NR^3SO_2-$, $-COCH_2-$, $-CH_2CO-$, $-COCR^3R^4-$, $-CR^3R^4CO-$, -NHCSNH-, $-NR^3CSNR^4$, -CH=CH-, $-CR^3=CR^4-$, or a heteroarylene group having 5 or 6 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, or a heteroalkylene group;

L^3 is absent or a bond, an oxygen atom, a sulphur atom or a group of formula NH, CONH, NHCO, COO, OCO, $CONR^3$, NR^3CO, OCONH, NHCOO, NHCONH, $OCONR^3$, NR^3COO, NR^3CONR^4, NR^3, $-CNR^3-$, -CO-, -SO-, $-SO_2-$, $-SO_2NH-$, $-NHSO_2-$, $-SO_2NR^3-$, $-NR^3SO_2-$, $-COCH_2-$, $-CH_2CO-$, $-COCR^3R^4-$, $-CR^3R^4CO-$, -NHCSNH-, $-NR^3CSNR^4$, -CH=CH-, $-CR^3=CR^4-$, or a heteroarylene group having 5 or 6 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, or a heteroalkylene group;

L^4 is absent or a bond, an oxygen atom, a sulphur atom or a group of formula NH, CONH, NHCO, COO, OCO, $CONR^3$, NR^3CO, OCONH, NHCOO, NHCONH, $OCONR^3$, NR^3COO, NR^3CONR^4, NR^3, $-CNR^3-$, -CO-, -SO-, $-SO_2-$, $-SO_2NH-$, $-NHSO_2-$, $-SO_2NR^3-$, $-NR^3SO_2-$, $-COCH_2-$, $-CH_2CO-$, $-COCR^3R^4-$, $-CR^3R^4CO-$, -

NHCSNH-, -NR^3CSNR^4, -CH=CH-, -$CR^3=CR^4$-, or a heteroarylene group having 5 or 6 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, or a heteroalkylene group;

R^1 is a hydrogen atom, a halogen atom, a hydroxy group, an amino group, a thiol group, a nitro group, a group of formula -COOH, -SO_2NH_2, -$CONH_2$, -NO_2 or -CN, an alkyl, an alkenyl, an alkynyl, a heteroalkyl, a cycloalkyl, a heterocycloalkyl, an alkylcycloalkyl, a heteroalkylcycloalkyl, an aryl, a heteroaryl, an aralkyl or a heteroaralkyl group;

R^2 is a hydrogen atom, a halogen atom, a hydroxy group, an amino group, a thiol group, a nitro group, a group of formula -COOH, -SO_2NH_2, -$CONH_2$, -NO_2 or -CN, an alkyl, an alkenyl, an alkynyl, a heteroalkyl, a cycloalkyl, a heterocycloalkyl, an alkylcycloalkyl, a heteroalkylcycloalkyl, an aryl, a heteroaryl, an aralkyl or a heteroaralkyl group;

the groups R^3 are independently from each other a hydrogen atom or a C_{1-6} alkyl group; and

the groups R^4 are independently from each other a hydrogen atom or a C_{1-6} alkyl group;

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

The expression alkyl refers to a saturated, straight-chain or branched hydrocarbon group that contains from 1 to 20 carbon atoms, preferably from 1 to 15 carbon atoms, especially from 1 to 10 (e.g. 1, 2, 3 or 4) carbon atoms, for example a methyl, ethyl, propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, tert-butyl, n-pentyl, iso-pentyl, n-hexyl, 2,2-dimethylbutyl or n-octyl group.

The expressions alkenyl and alkynyl refer to at least partially unsaturated, straight-

chain or branched hydrocarbon groups that contain from 2 to 20 carbon atoms, preferably from 2 to 15 carbon atoms, especially from 2 to 10 (e.g. 2, 3 or 4) carbon atoms, for example an ethenyl (vinyl), propenyl (allyl), iso-propenyl, butenyl, ethinyl, propinyl, butinyl, acetylenyl, propargyl, isoprenyl or hex-2-enyl group. Preferably, alkenyl groups have one or two (especially preferably one) double bond(s), and alkynyl groups have one or two (especially preferably one) triple bond(s).

Furthermore, the terms alkyl, alkenyl and alkynyl refer to groups in which one or more hydrogen atoms have been replaced by a halogen atom (preferably F or Cl) such as, for example, a 2,2,2-trichloroethyl or a trifluoromethyl group.

The expression heteroalkyl refers to an alkyl, alkenyl or alkynyl group in which one or more (preferably 1 to 8; especially preferably 1, 2, 3 or 4) carbon atoms have been replaced by an oxygen, nitrogen, phosphorus, boron, selenium, silicon or sulfur atom (preferably by an oxygen, sulfur or nitrogen atom) or by a SO or a SO_2 group. The expression heteroalkyl furthermore refers to a carboxylic acid or to a group derived from a carboxylic acid, such as, for example, acyl, acylalkyl, alkoxycarbonyl, acyloxy, acyloxyalkyl, carboxyalkylamide or alkoxycarbonyloxy.

Preferably, a heteroalkyl group contains from 1 to 12 carbon atoms and from 1 to 8 heteroatoms selected from oxygen, nitrogen and sulphur (especially oxygen and nitrogen). Especially preferably, a heteroalkyl group contains from 1 to 6 (e.g. 1, 2, 3 or 4) carbon atoms and 1, 2, 3 or 4 (especially 1, 2 or 3) heteroatoms selected from oxygen, nitrogen and sulphur (especially oxygen and nitrogen). The term C_1-C_6 heteroalkyl refers to a heteroalkyl group containing from 1 to 6 carbon atoms and 1, 2 or 3 heteroatoms selected from O, S and/or N (especially O and/or N). The term C_1-C_4 heteroalkyl refers to a heteroalkyl group containing from 1 to 4 carbon atoms and 1, 2 or 3 heteroatoms selected from O, S and/or N (especially O and/or N). Furthermore, the term heteroalkyl refers to groups in which one or more hydrogen atoms have been replaced by a halogen atom (preferably F or Cl).

Especially preferably, the expression heteroalkyl refers to an alkyl group as defined

above (straight-chain or branched) in which one or more (preferably 1 to 6; especially preferably 1, 2, 3 or 4) carbon atoms have been replaced by an oxygen, sulfur or nitrogen atom; this group preferably contains from 1 to 6 (e.g. 1, 2, 3 or 4) carbon atoms and 1, 2, 3 or 4 (especially 1, 2 or 3) heteroatoms selected from oxygen, nitrogen and sulphur (especially oxygen and nitrogen); this group may preferably be substituted by one or more (preferably 1 to 6; especially preferably 1, 2, 3 or 4) fluorine, chlorine, bromine or iodine atoms or OH, =O, SH, =S, NH_2, =NH, N_3, CN or NO_2 groups.

The expression heteroalkylene group refers to a divalent heteroalkyl group.

Examples of heteroalkyl groups are groups of formulae: R^a-O-Y^a-, R^a-S-Y^a-, R^a-SO-Y^a-, R^a-SO_2-Y^a-, R^a-N(R^b)-Y^a-, R^a-CO-Y^a-, R^a-O-CO-Y^a-, R^a-CO-O-Y^a-, R^a-CO-N(R^b)-Y^a-, R^a-N(R^b)-CO-Y^a-, R^a-O-CO-N(R^b)-Y^a-, R^a-N(R^b)-CO-O-Y^a-, R^a-N(R^b)-CO-N(R^c)-Y^a-, R^a-O-CO-O-Y^a-, R^a-N(R^b)-C(=NR^d)-N(R^c)-Y^a-, R^a-CS-Y^a-, R^a-O-CS-Y^a-, R^a-CS-O-Y^a-, R^a-CS-N(R^b)-Y^a-, R^a-N(R^b)-CS-Y^a-, R^a-O-CS-N(R^b)-Y^a-, R^a-N(R^b)-CS-O-Y^a-, R^a-N(R^b)-CS-N(R^c)-Y^a-, R^a-O-CS-O-Y^a-, R^a-S-CO-Y^a-, R^a-CO-S-Y^a-, R^a-S-CO-N(R^b)-Y^a-, R^a-N(R^b)-CO-S-Y^a-, R^a-S-CO-O-Y^a-, R^a-O-CO-S-Y^a-, R^a-S-CO-S-Y^a-, R^a-S-CS-Y^a-, R^a-CS-S-Y^a-, R^a-S-CS-N(R^b)-Y^a-, R^a-N(R^b)-CS-S-Y^a-, R^a-S-CS-O-Y^a-, R^a-O-CS-S-Y^a-, wherein R^a being a hydrogen atom, a C_1-C_6 alkyl, a C_2-C_6 alkenyl or a C_2-C_6 alkynyl group; R^b being a hydrogen atom, a C_1-C_6 alkyl, a C_2-C_6 alkenyl or a C_2-C_6 alkynyl group; R^c being a hydrogen atom, a C_1-C_6 alkyl, a C_2-C_6 alkenyl or a C_2-C_6 alkynyl group; R^d being a hydrogen atom, a C_1-C_6 alkyl, a C_2-C_6 alkenyl or a C_2-C_6 alkynyl group and Y^a being a bond, a C_1-C_6 alkylene, a C_2-C_6 alkenylene or a C_2-C_6 alkynylene group, wherein each heteroalkyl group contains at least one carbon atom and one or more hydrogen atoms may be replaced by fluorine or chlorine atoms.

Specific examples of heteroalkyl groups are methoxy, trifluoromethoxy, ethoxy, n-propyloxy, isopropyloxy, butoxy, *tert*-butyloxy, methoxymethyl, ethoxymethyl, -CH_2CH_2OH, -CH_2OH, -SO_2Me, methoxyethyl, 1-methoxyethyl, 1-ethoxyethyl, 2-methoxyethyl or 2-ethoxyethyl, methylamino, ethylamino, propylamino,

isopropylamino, dimethylamino, diethylamino, isopropylethylamino, methylamino methyl, ethylamino methyl, diisopropylamino ethyl, methylthio, ethylthio, isopropylthio, enol ether, dimethylamino methyl, dimethylamino ethyl, acetyl, propionyl, butyryloxy, acetyloxy, methoxycarbonyl, ethoxycarbonyl, propionyloxy, acetylamino or propionylamino, carboxymethyl, carboxyethyl or carboxypropyl, N-ethyl-N-methylcarbamoyl or N-methylcarbamoyl. Further examples of heteroalkyl groups are nitrile, isonitrile, cyanate, thiocyanate, isocyanate, isothiocyanate and alkylnitrile groups.

The expression cycloalkyl refers to a saturated or partially unsaturated (for example, a cycloalkenyl group) cyclic group that contains one or more rings (preferably 1 or 2), and contains from 3 to 14 ring carbon atoms, preferably from 3 to 10 (especially 3, 4, 5, 6 or 7) ring carbon atoms. The expression cycloalkyl refers furthermore to groups in which one or more hydrogen atoms have been replaced by fluorine, chlorine, bromine or iodine atoms or by OH, =O, SH, =S, NH_2, =NH, N_3 or NO_2 groups, thus, for example, cyclic ketones such as, for example, cyclohexanone, 2-cyclohexenone or cyclopentanone. Further specific examples of cycloalkyl groups are a cyclopropyl, cyclobutyl, cyclopentyl, spiro[4,5]decanyl, norbornyl, cyclohexyl, cyclopentenyl, cyclohexadienyl, decalinyl, bicyclo[4.3.0]nonyl, tetraline, cyclopentylcyclohexyl, fluorocyclohexyl or cyclohex-2-enyl group.

The expression heterocycloalkyl refers to a cycloalkyl group as defined above in which one or more (preferably 1, 2 or 3) ring carbon atoms have been replaced by an oxygen, nitrogen, silicon, selenium, phosphorus or sulfur atom (preferably by an oxygen, sulfur or nitrogen atom) or a SO group or a SO_2 group. A heterocycloalkyl group has preferably 1 or 2 ring(s) containing from 3 to 10 (especially 3, 4, 5, 6 or 7) ring atoms (preferably secected from C, O, N and S). The expression heterocyclo-alkyl refers furthermore to groups that are substituted by fluorine, chlorine, bromine or iodine atoms or by OH, =O, SH, =S, NH_2, =NH, N_3 or NO_2 groups. Examples are a piperidyl, prolinyl, imidazolidinyl, piperazinyl, morpholinyl, urotropinyl, pyrrolidinyl, tetrahydrothiophenyl, tetrahydropyranyl, tetrahydrofuryl or 2-pyrazolinyl group and also lactames, lactones, cyclic imides and cyclic anhydrides.

The expression alkylcycloalkyl refers to groups that contain both cycloalkyl and also alkyl, alkenyl or alkynyl groups in accordance with the above definitions, for example alkylcycloalkyl, cycloalkylalkyl, alkylcycloalkenyl, alkenylcycloalkyl and alkynylcycloalkyl groups. An alkylcycloalkyl group preferably contains a cycloalkyl group that contains one or two rings having from 3 to 10 (especially 3, 4, 5, 6 or 7) ring carbon atoms, and one or two alkyl, alkenyl or alkynyl groups (especially alkyl groups) having 1 or 2 to 6 carbon atoms.

The expression heteroalkylcycloalkyl refers to alkylcycloalkyl groups as defined above in which one or more (preferably 1, 2 or 3) carbon atoms have been replaced by an oxygen, nitrogen, silicon, selenium, phosphorus or sulfur atom (preferably by an oxygen, sulfur or nitrogen atom) or a SO group or a SO_2 group. A heteroalkylcycloalkyl group preferably contains 1 or 2 rings having from 3 to 10 (especially 3, 4, 5, 6 or 7) ring atoms, and one or two alkyl, alkenyl, alkynyl or heteroalkyl groups (especially alkyl or heteroalkyl groups) having from 1 or 2 to 6 carbon atoms. Examples of such groups are alkylheterocycloalkyl, alkylheterocycloalkenyl, alkenylheterocycloalkyl, alkynylheterocycloalkyl, heteroalkylcycloalkyl, heteroalkylheterocycloalkyl and heteroalkylheterocycloalkenyl, the cyclic groups being saturated or mono-, di- or tri-unsaturated.

The expression aryl refers to an aromatic group that contains one or more rings containing from 6 to 14 ring carbon atoms, preferably from 6 to 10 (especially 6) ring carbon atoms. The expression aryl refers furthermore to groups that are substituted by fluorine, chlorine, bromine or iodine atoms or by OH, SH, NH_2, N_3 or NO_2 groups. Examples are the phenyl, naphthyl, biphenyl, 2-fluorophenyl, anilinyl, 3-nitrophenyl or 4-hydroxyphenyl group.

The expression heteroaryl refers to an aromatic group that contains one or more rings containing from 5 to 14 ring atoms, preferably from 5 to 10 (especially 5 or 6 or 9 or 10) ring atoms, and contains one or more (preferably 1, 2, 3 or 4) oxygen, nitrogen, phosphorus or sulfur ring atoms (preferably O, S or N). The expression

heteroaryl refers furthermore to groups that are substituted by fluorine, chlorine, bromine or iodine atoms or by OH, SH, N_3, NH_2 or NO_2 groups. Examples are pyridyl (e.g. 4-pyridyl), imidazolyl (e.g. 2-imidazolyl), phenylpyrrolyl (e.g. 3-phenylpyrrolyl), thiazolyl, isothiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, oxadiazolyl,thiadiazolyl, indolyl, indazolyl, tetrazolyl, pyrazinyl, pyrimidinyl, pyridazinyl, oxazolyl, isoxazolyl, triazolyl, tetrazolyl, isoxazolyl, indazolyl, indolyl, benzimidazolyl, benzoxazolyl, benzisoxazolyl, benzthiazolyl, pyridazinyl, quinolinyl, isoquinolinyl, pyrrolyl, purinyl, carbazolyl, acridinyl, pyrimidyl, 2,3′-bifuryl, pyrazolyl (e.g. 3-pyrazolyl) and isoquinolinyl groups.

The expression aralkyl refers to groups containing both aryl and also alkyl, alkenyl, alkynyl and/or cycloalkyl groups in accordance with the above definitions, such as, for example, arylalkyl, arylalkenyl, arylalkynyl, arylcycloalkyl, arylcycloalkenyl, alkylarylcycloalkyl and alkylarylcycloalkenyl groups. Specific examples of aralkyls are toluene, xylene, mesitylene, styrene, benzyl chloride, o-fluorotoluene, 1H-indene, tetraline, dihydronaphthalene, indanone, phenylcyclopentyl, cumene, cyclohexylphenyl, fluorene and indane. An aralkyl group preferably contains one or two aromatic ring systems (especially 1 or 2 rings), each containing from 6 to 10 carbon atoms and one or two alkyl, alkenyl and/or alkynyl groups containing from 1 or 2 to 6 carbon atoms and/or a cycloalkyl group containing 5 or 6 ring carbon atoms.

The expression heteroaralkyl refers to groups containing both aryl or heteroaryl, respectively, and also alkyl, alkenyl, alkynyl and/or heteroalkyl and/or cycloalkyl and/or heterocycloalkyl groups in accordance with the above definitions. A hetero-aralkyl group preferably contains one or two aromatic ring systems (especially 1 or 2 rings), each containing from 5 or 6 to 9 or 10 ring carbon atoms and one or two alkyl, alkenyl and/or alkynyl groups containing 1 or 2 to 6 carbon atoms and/or one or two heteroalkyl groups containing 1 to 6 carbon atoms and 1, 2 or 3 heteroatoms selected from O, S and N and/or one or two cycloalkyl groups each containing 5 or 6 ring carbon atoms and/or one or two heterocycloalkyl groups, each containing 5 or 6 ring atoms comprising 1, 2, 3 or 4 oxygen, sulfur or nitrogen atoms.

Examples are arylheteroalkyl, arylheterocycloalkyl, arylheterocycloalkenyl, arylalkyl-

heterocycloalkyl, arylalkenylheterocycloalkyl, arylalkynylheterocycloalkyl, arylalkyl-heterocycloalkenyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heteroaryl-heteroalkyl, heteroarylcycloalkyl, heteroarylcycloalkenyl, heteroarylheterocycloalkyl, heteroarylheterocycloalkenyl, heteroarylalkylcycloalkyl, heteroarylalkylheterocyclo-alkenyl, heteroarylheteroalkylcycloalkyl, heteroarylheteroalkylcycloalkenyl and heteroarylheteroalkylheterocycloalkyl groups, the cyclic groups being saturated or mono-, di- or tri-unsaturated. Specific examples are a tetrahydroisoquinolinyl, benzoyl, 2- or 3-ethylindolyl, 4-methylpyridino, 2-, 3- or 4-methoxyphenyl, 4-ethoxy-phenyl, 2-, 3- or 4-carboxyphenylalkyl group.

As already stated above, the expressions cycloalkyl, heterocycloalkyl, alkylcycloalkyl, heteroalkylcycloalkyl, aryl, heteroaryl, aralkyl and heteroaralkyl also refer to groups that are substituted by fluorine, chlorine, bromine or iodine atoms or by OH, =O, SH, =S, NH_2, =NH, N_3 or NO_2 groups.

The expression "optionally substituted" especially refers to groups that are optionally substituted by fluorine, chlorine, bromine or iodine atoms or by OH, =O, SH, =S, NH_2, =NH, N_3 or NO_2 groups. This expression refers furthermore to groups that may be substituted by one, two, three or more unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} heteroalkyl, C_3-C_{18} cycloalkyl, C_2-C_{17} heterocycloalkyl, C_4-C_{20} alkylcycloalkyl, C_2-C_{19} heteroalkylcycloalkyl, C_6-C_{18} aryl, C_1-C_{17} heteroaryl, C_7-C_{20} aralkyl or C_2-C_{19} heteroaralkyl groups. This expression refers furthermore especially to groups that may be substituted by one, two, three or more unsubstituted C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_1-C_6 heteroalkyl, C_3-C_{10} cycloalkyl, C_2-C_9 heterocycloalkyl, C_7-C_{12} alkylcycloalkyl, C_2-C_{11} heteroalkylcycloalkyl, C_6-C_{10} aryl, C_1-C_9 heteroaryl, C_7-C_{12} aralkyl or C_2-C_{11} heteroaralkyl groups.

Especially preferably at group Ar^1, Ar^2, Ar^3, Ar^4 and Ar^5, the expression "optionally substituted" refers to groups that are optionally substituted by one, two or three groups independently selected from halogen atoms, hydroxy groups, groups of formula -O-alkyl (e.g. -O-C_{1-6} alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), $-NH_2$, $-NR^{5a}R^{6a}$ (wherein R^{5a} and R^{6a} independently from each other

are a hydrogen atom or an alkyl group such as a C_{1-6} alkyl group), $-SO_2NH_2$, $-CONH_2$, -CN, -alkyl (e.g. $-C_{1-6}$ alkyl, $-CF_3$), -SH, -S-alkyl (e.g. $-S-C_{1-6}$ alkyl).

Most preferably at group Ar^1, Ar^2, Ar^3, Ar^4 and Ar^5, the expression "optionally substituted" refers to groups that are optionally substituted by one, two or three groups independently selected from F, Cl, hydroxy groups, groups of formula $-O-C_{1-6}$ alkyl (especially $-O-C_{1-4}$ alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), and $-C_{1-6}$ alkyl (e.g. $-C_{1-4}$ alkyl such as $-CH_3$ or $-CF_3$).

Especially preferably at group Ar^6, the expression "optionally substituted" refers to groups that are optionally substituted by one, two or three groups independently selected from halogen atoms, hydroxy groups, groups of formula -O-alkyl (e.g. $-O-C_{1-6}$ alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), $-NH_2$, $-NR^{5a}R^{6a}$ (wherein R^{5a} and R^{6a} independently from each other are a hydrogen atom or an alkyl group such as a C_{1-6} alkyl group), $-SO_2NH_2$, $-CONH_2$, -CN, -alkyl (e.g. $-C_{1-6}$ alkyl, $-CF_3$), -SH, -S-alkyl (e.g. $-S-C_{1-6}$ alkyl) and NO_2.

Most preferably at group Ar^6, the expression "optionally substituted" refers to groups that are optionally substituted by one, two or three groups independently selected from F, Cl, hydroxy groups, $-NH_2$, $-NO_2$, groups of formula $-O-C_{1-6}$ alkyl (especially $-O-C_{1-4}$ alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), and $-C_{1-6}$ alkyl (e.g. $-C_{1-4}$ alkyl such as $-CH_3$ or $-CF_3$).

The term halogen refers to F, Cl, Br or I.

According to a preferred embodiment, all alkyl, alkenyl, alkynyl, heteroalkyl, aryl, heteroaryl, cycloalkyl, heterocycloalkyl, alkylcycloalkyl, heteroalkylcycloalkyl, aralkyl and heteroaralkyl groups described herein may independently of each other optionally be substituted.

When an aryl, heteroaryl, cycloalkyl, alkylcycloalkyl, heteroalkylcycloalkyl, heterocycloalkyl, aralkyl or heteroaralkyl group contains more than one ring, these

rings may be bonded to each other via a single or double bond or these rings may be annulated.

Owing to their substitution, compounds of formula (I) may contain one or more centers of chirality. The present invention therefore includes both all pure enantiomers and all pure diastereoisomers and also mixtures thereof in any mixing ratio. The present invention moreover also includes all cis/trans-isomers of the compounds of the general formula (I) and also mixtures thereof. The present invention moreover includes all tautomeric forms of the compounds of formula (I).

Preferably, when Ar^4 is absent, also L^3 is absent.

Further preferably, when Ar^5 is absent, also L^4 is absent.

Preferably, Ar^1 is an optionally substituted 1, 4-phenylene group or an optionally substituted 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen.

Further preferably, Ar^1 is an optionally substituted 1,4-phenylene group.

Preferably, Ar^2 is an optionally substituted 1,4-phenylene group or an optionally substituted 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen.

Further preferably, Ar^2 is an optionally substituted 1,4-phenylene group.

Preferably, Ar^3 is an optionally substituted 1,4-phenylene group or an optionally substituted 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen.

Further preferably, Ar^3 is an optionally substituted 1,4-phenylene group.

Preferably, Ar^4 is an optionally substituted 1,4-phenylene group or an optionally substituted 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen.

Further preferably, Ar^4 is an optionally substituted 1,4-phenylene group.

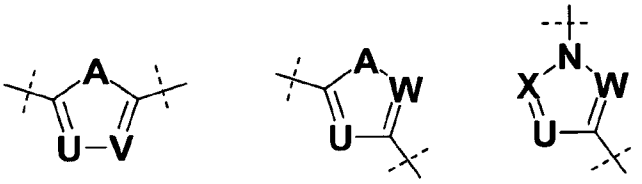
Preferably, Ar^5 is an optionally substituted 1,4-phenylene group or an optionally substituted 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen.

Further preferably, Ar^5 is an optionally substituted 1,4-phenylene group.

Further preferably, Ar^4 is absent.

Further preferably, Ar^5 is absent.

The term 1,3-heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen especially preferably refers to one of the following groups:



wherein A is O, S or NH; U is N or CH; V is N or CH; W is N or CH; and X is N or CH.

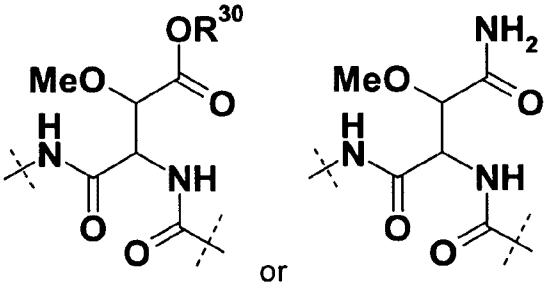
Further preferably, L^1 is a group of formula -CONH-, -NHCO-, -SO_2NH-, -NHSO_2-, -CH=CH-, -CR^3=CR^4- or an optionally substituted heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, wherein R^3 and R^4 are independently from each other a C_{1-6} alkyl group.

Further preferably, L^2 is a group of formula -CONH-, -NHCO-, $-SO_2NH-$, $-NHSO_2-$, -CH=CH-, $-CR^3=CR^4-$ or an optionally substituted heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, wherein R^3 and R^4 are independently from each other a C_{1-6} alkyl group.

Further preferably, L^3 is absent or a group of formula -CONH-, -NHCO-, $-SO_2NH-$, $-NHSO_2-$, -CH=CH-, $-CR^3=CR^4-$ or an optionally substituted heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, wherein R^3 and R^4 are independently from each other a C_{1-6} alkyl group.

Further preferably, L^4 is absent or a group of formula -CONH-, -NHCO-, $-SO_2NH-$, $-NHSO_2-$, -CH=CH-, $-CR^3=CR^4-$ or an optionally substituted heteroarylene group having 5 ring atoms including 1, 2, or 3 heteroatoms selected from oxygen, sulphur and nitrogen, wherein R^3 and R^4 are independently from each other a C_{1-6} alkyl group.

Further preferably, L^1 is NHCO (wherein the nitrogen atom is bound to Ar^1) or a group of the following formula:

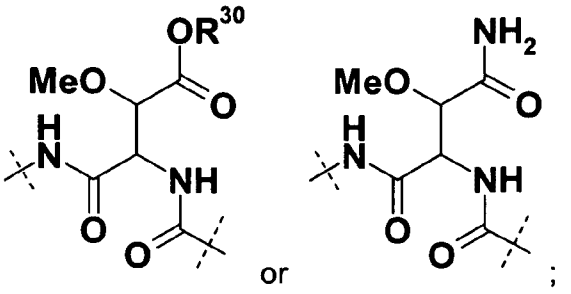


or

(wherein the NH group is bound to Ar^1), wherein R^{30} is a hydrogen atom or a C_{1-3} alkyl group.

Especially preferably, L^1 is NHCO (wherein the nitrogen atom is bound to Ar^1).

Moreover preferably, L^2 is NHCO (wherein the nitrogen atom is bound to Ar^2) or a group of the following formula:

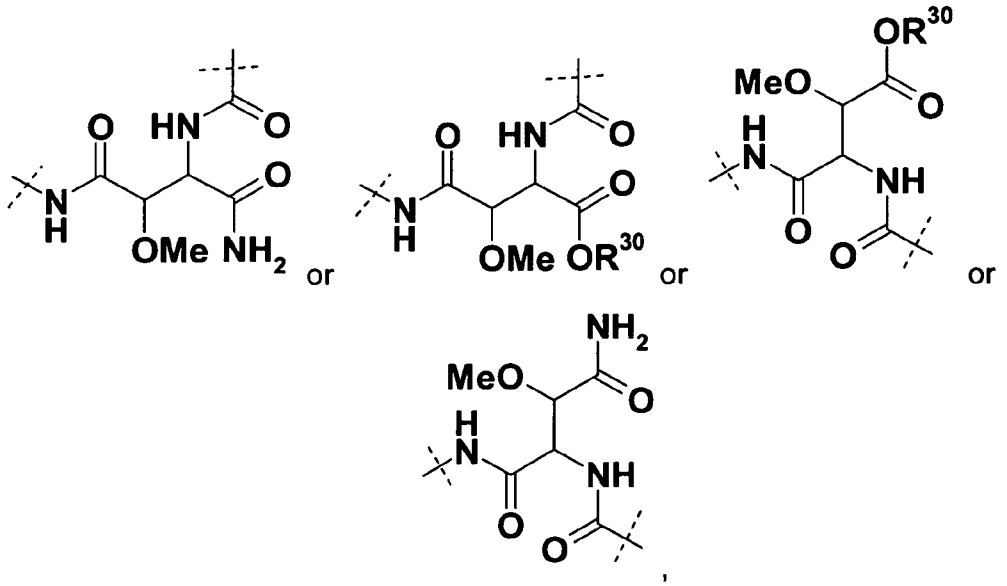


;

(wherein the NH group is bound to Ar^2), wherein R^{30} is a hydrogen atom or a C_{1-3} alkyl group.

Especially preferably, L^2 is NHCO (wherein the nitrogen atom is bound to Ar^1).

Further preferably, L^3 is absent or a group of the following formula:



,

(wherein the NH group is bound to Ar^3), wherein R^{30} is a hydrogen atom or a C_{1-3}alkyl group.

Further preferably, L^4 is absent or NHCO (wherein the nitrogen atom is bound to Ar^4).

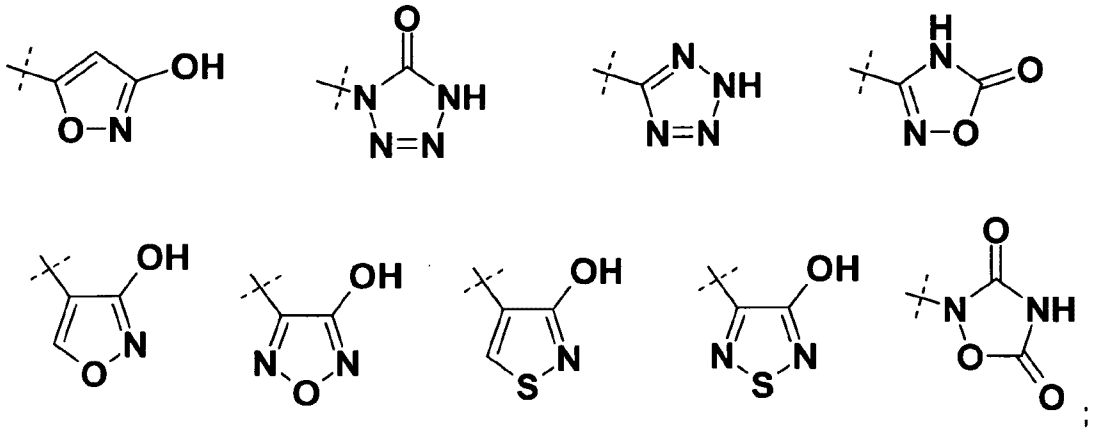
Moreover preferably, R^{30} is a hydrogen atom.

Further preferably, R^1 is a hydrogen atom, a halogen atom or a group of formula -OH,

-NH_2, -COOH, -SO_2NH_2, -$CONH_2$, -NO_2, -CN, -alkyl (e.g. -CF_3), -O-alkyl, -O-CO-alkyl, -NH-alkyl, -NH-CO-alkyl, or an optionally substituted heteroaryl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen, or an optionally substituted heterocycloalkyl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen.

Moreover preferably, R^2 is a hydrogen atom, a halogen atom or a group of formula -OH, -NH_2, -COOH, -SO_2NH_2, -$CONH_2$, -NO_2, -CN, -alkyl (e.g. -CF_3), -O-alkyl, -O-CO-alkyl, -NH-alkyl, -NH-CO-alkyl, or an optionally substituted heteroaryl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen, or an optionally substituted heterocycloalkyl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen.

Preferred examples of optionally substituted heteroaryl groups having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen and of optionally substituted heterocycloalkyl groups having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen as groups R^1 and R^2 are isosteres of carboxylic acid such as groups of the following formulas:



all these groups may optionally be further substituted.

Especially preferably, R^1 is a group of formula -NH_2, -NO_2, $COOR^{11}$, or -$CONR^{12}R^{13}$; wherein R^{11}, R^{12} and R^{13} are independently a hydrogen atom or a C_{1-6} alkyl group; moreover preferably, R^1 is a group of formula -COOH.

Further especially preferably, R^2 is a group of formula $-NH_2$, $-NO_2$, $COOR^{11a}$, or $-CONR^{12a}R^{13a}$; wherein R^{11a}, R^{12a} and R^{13a} are independently a hydrogen atom or a C_{1-6} alkyl group; moreover preferably, R^2 is a group of formula $-NH_2$ or $-NO_2$.

Further especially preferably, R^1 is a heteroaryl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen, and which is substituted by a hydroxy group.

Further especially preferably, R^2 is a heteroaryl group having 5 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen, and which is substituted by a hydroxy group.

Especially preferred are compounds of formula (I)

$$R^1\text{-}Ar^1\text{-}L^1\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

(I)

wherein

Ar^1 is an optionally substituted 1,4-phenylene group;

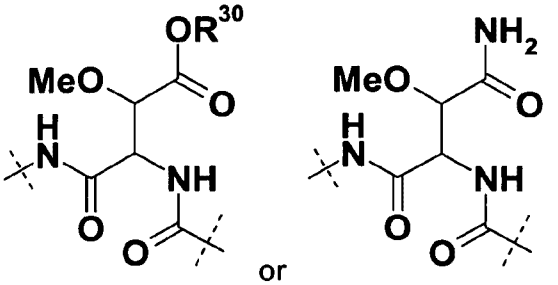
Ar^2 is an optionally substituted 1,4-phenylene group;

Ar^3 is an optionally substituted 1,4-phenylene group;

Ar^4 is absent or an optionally substituted 1,4-phenylene group;

Ar^5 is absent or an optionally substituted 1,4-phenylene group;

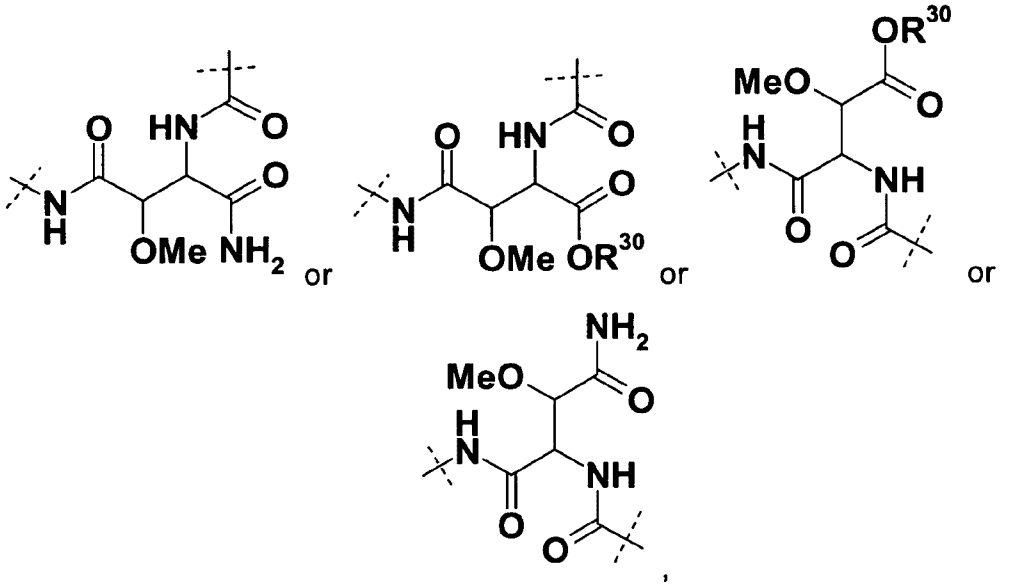
L^1 is a group of formula -CONH-, -NHCO-, $-SO_2NH-$ or $-NHSO_2-$ or a group of the following formula:



(wherein the NH group is bound to Ar^1);

L^2 is a group of formula -CONH-, -NHCO-, -SO_2NH- or -$NHSO_2$-;

L^3 is absent or a group of formula -CONH-, -NHCO-, -SO_2NH- or -$NHSO_2$- or a group of the following formula:



(wherein the NH group is bound to Ar^3);

L^4 is absent or a group of formula -CONH-, -NHCO-, -SO_2NH- or -$NHSO_2$-;

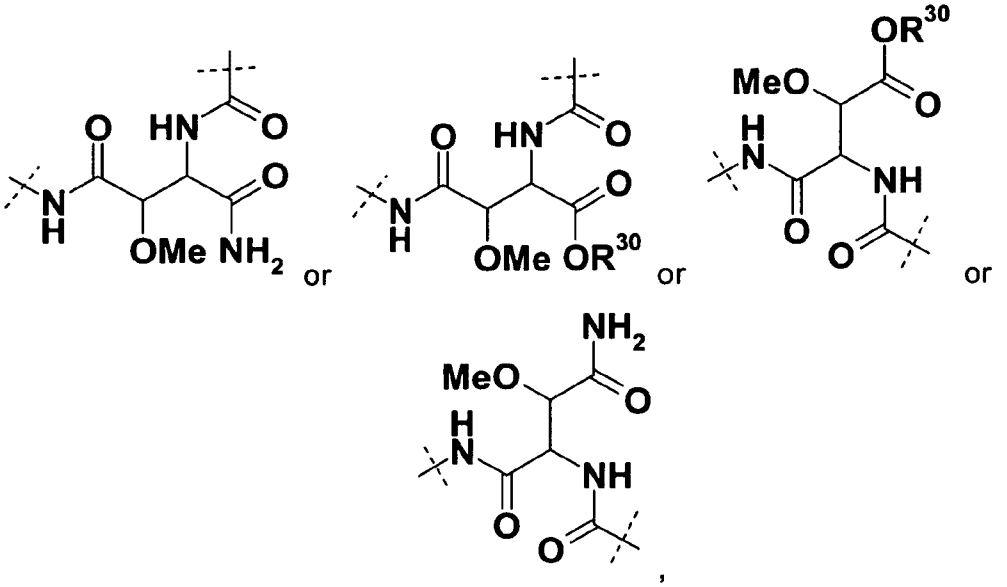
R^{30} is a hydrogen atom or a C_{1-3} alkyl group (especially preferably, a hydrogen atom);

R^1 is a group of formula -NH_2, -NO_2, $COOR^{11}$, or -$CONR^{12}R^{13}$; wherein R^{11}, R^{12} and R^{13} are independently a hydrogen atom or a C_{1-6} alkyl group (especially preferably, R^1 is a group of formula -COOH); and

R^2 is a group of formula -NH_2, -NO_2, $COOR^{11a}$, or -$CONR^{12a}R^{13a}$; wherein R^{11a}, R^{12a} and R^{13a} are independently a hydrogen atom or a C_{1-6} alkyl group (especially preferably, R^2 is a group of formula -NH_2 or -NO_2);

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

Therein, preferably, L^1 is a group of formula -CONH-, -NHCO-, -SO_2NH- or -$NHSO_2$-, and L^3 is absent or a group of the following formula:



(wherein the NH group is bound to Ar^3).

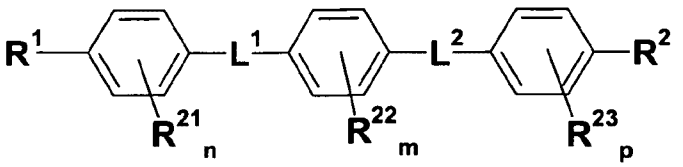
Further preferred are compounds of formula (II)

$$R^1\text{-}Ar^1\text{-}L^1\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}R^2$$

(II)

wherein Ar^1, Ar^2, Ar^3, L^1, L^2, R^1 and R^2 are as defined above.

Moreover preferred are compounds of formula (III)



(III)

wherein

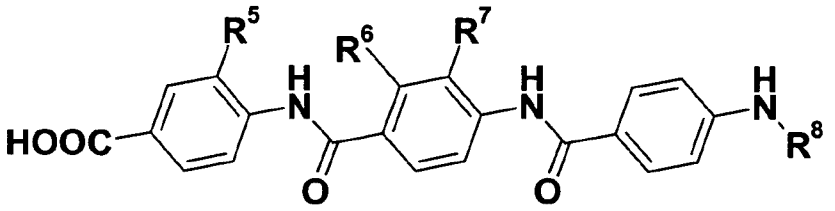
n is 0, 1, 2, 3 or 4;

m is 0, 1, 2, 3 or 4;

p is 0, 1, 2, 3 or 4;

group(s) R^{21} are independently selected from halogen atoms, hydroxy groups, groups of formula -O-alkyl (e.g. -O-C_{1-6} alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), $-NH_2$, $-NR^{5a}R^{6a}$ (wherein R^{5a} and R^{6a} independently from each other are a hydrogen atom or an alkyl group such as a C_{1-6} alkyl group), $-SO_2NH_2$, $-CONH_2$, -CN, -alkyl (e.g. -C_{1-6} alkyl, $-CF_3$), -SH, -S-alkyl (e.g. -S-C_{1-6} alkyl);
group(s) R^{22} are independently selected from halogen atoms, hydroxy groups, groups of formula -O-alkyl (e.g. -O-C_{1-6} alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), $-NH_2$, $-NR^{5a}R^{6a}$ (wherein R^{5a} and R^{6a} independently from each other are a hydrogen atom or an alkyl group such as a C_{1-6} alkyl group), $-SO_2NH_2$, $-CONH_2$, -CN, -alkyl (e.g. -C_{1-6} alkyl, $-CF_3$), -SH, -S-alkyl (e.g. -S-C_{1-6} alkyl);
group(s) R^{23} are independently selected from halogen atoms, hydroxy groups, groups of formula -O-alkyl (e.g. -O-C_{1-6} alkyl such as -OMe, -OEt, -O-nPr, -O-iPr, -O-nBu, -O-iBu or -O-tBu), $-NH_2$, $-NR^{5a}R^{6a}$ (wherein R^{5a} and R^{6a} independently from each other are a hydrogen atom or an alkyl group such as a C_{1-6} alkyl group), $-SO_2NH_2$, $-CONH_2$, -CN, -alkyl (e.g. -C_{1-6} alkyl, $-CF_3$), -SH, -S-alkyl (e.g. -S-C_{1-6} alkyl);
and
R^1, R^2, L^1 and L^2 are as defined above.

Further preferred are compounds of formula (IV)

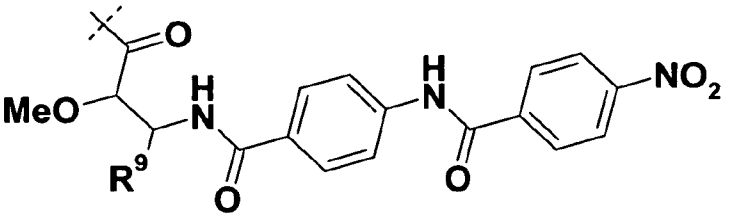


(IV)

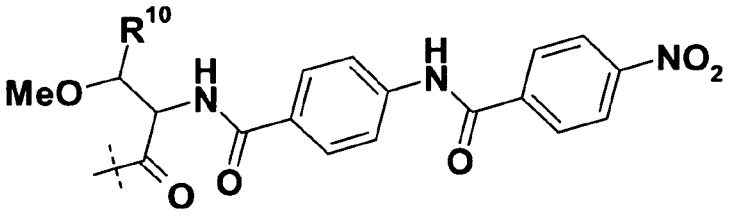
wherein
R^5 is a group of formula -O-C_{1-6} alkyl;
R^6 is a hydroxy group;
R^7 is a group of formula -O-C_{1-6} alkyl; and
R^8 is a hydrogen atom, an alkyl, an alkenyl, an alkynyl, a heteroalkyl, a cycloalkyl, a heterocycloalkyl, an alkylcycloalkyl, a heteroalkylcycloalkyl, an aryl, a heteroaryl, an

aralkyl or a heteroaralkyl group.

Preferably, R^8 is a hydrogen atom or a group of the following formula:



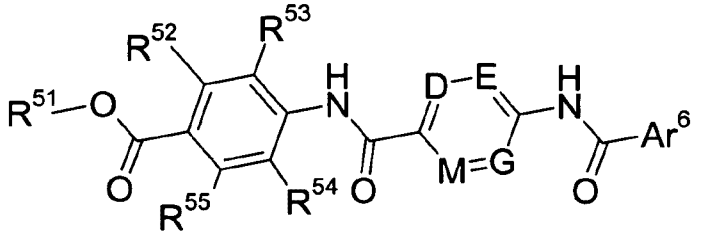
or



wherein R^9 is COOH or $CONH_2$ and R^{10} is COOH or $CONH_2$.

Moreover preferably, R^5 is a group of formula -O-C_{1-4} alkyl and R^7 is a group of formula -O-C_{1-4} alkyl.

Further preferred are compounds of formula (V)



(V)

wherein

R^{51} is a hydrogen atom, or a C_{1-6} alkyl group;

R^{52} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{53} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{54} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{55} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
D is N or CR^{56};
E is N or CR^{57};
G is N or CR^{58};
M is N or CR^{59};
R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl; and
Ar^6 is an optionally substituted (by one, two or more substituents such as e.g. R^2, R^8 or NHR^8) phenyl group or an optionally substituted (by one, two or more substituents such as e.g. R^2, R^8 or NHR^8) heteroaryl group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;
or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

Especially preferred are compounds of Formula (V) wherein:
R^{51} is a hydrogen atom, or a C_{1-4} alkyl group;
R^{52} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{53} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{54} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of

formula -O-C_{1-4} alkyl;

R^{55} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;

D is N or CR^{56};

E is N or CR^{57};

G is N or CR^{58};

M is N or CR^{59};

R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;

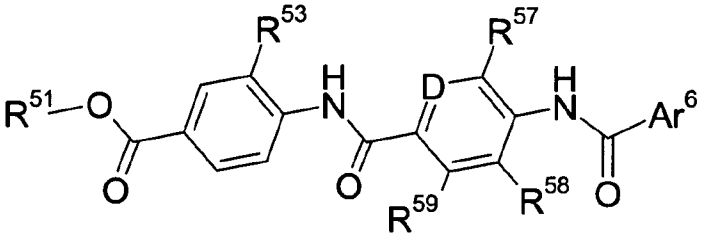
R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;

R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl; and

R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-4} alkyl.

Especially preferably, only one or two (especially only one) of D, E, G and M is/are N.

Further preferred are compounds of formula (VI)



(VI)

wherein

R^{51} is a hydrogen atom, or a C_{1-6} alkyl group;

R^{53} is F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl (especially preferably a group of formula -O-C_{1-6} alkyl);

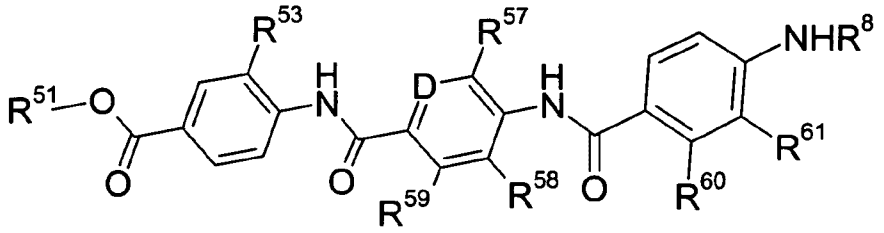
D is N or CR^{56};

R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of

formula -O-C_{1-6} alkyl;
R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;
R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl; and
Ar^6 is an optionally substituted (by one, two or more substituents such as e.g. R^2, R^8 or NHR^8) phenyl group or an optionally substituted (by one, two or more substituents such as e.g. R^2, R^8 or NHR^8) heteroaryl group having 5 or 6 ring atoms including 1, 2, 3 or 4 heteroatoms selected from oxygen, sulphur and nitrogen;
or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

Especially preferred are compounds of Formula (VI) wherein:
R^{51} is a hydrogen atom, or a C_{1-4} alkyl group;
R^{53} is F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl (especially preferably a group of formula -O-C_{1-4} alkyl);
D is N or CR^{56};
R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl; and
R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl.

Further preferred are compounds of formula (VII)



(VII)

wherein

R^{51} is a hydrogen atom, or a C_{1-6} alkyl group;

R^{53} is F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl (especially preferably a group of formula -O-C_{1-6} alkyl);

D is N or CR^{56};

R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{60} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{61} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl; and

R^{8} is a hydrogen atom, an alkyl, an alkenyl, an alkynyl, a heteroalkyl, a cycloalkyl, a heterocycloalkyl, an alkylcycloalkyl, a heteroalkylcycloalkyl, an aryl, a heteroaryl, an aralkyl or a heteroaralkyl group.

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

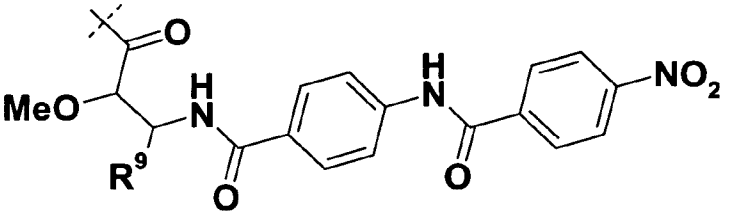
Especially preferred are compounds of Formula (VII) wherein:

R^{51} is a hydrogen atom, or a C_{1-4} alkyl group;

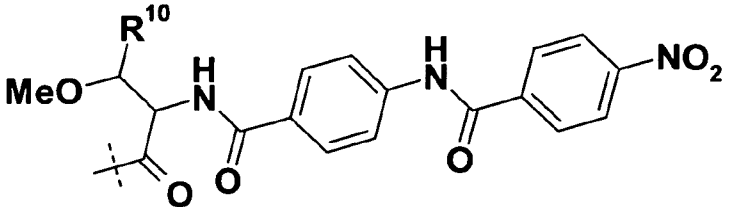
R^{53} is F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl

(especially preferably a group of formula -O-C_{1-4} alkyl);
D is N or CR^{56};
R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl;
R^{60} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl; and
R^{61} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-4} alkyl group or a group of formula -O-C_{1-4} alkyl.

Preferably, R^8 is a hydrogen atom or a group of the following formula:

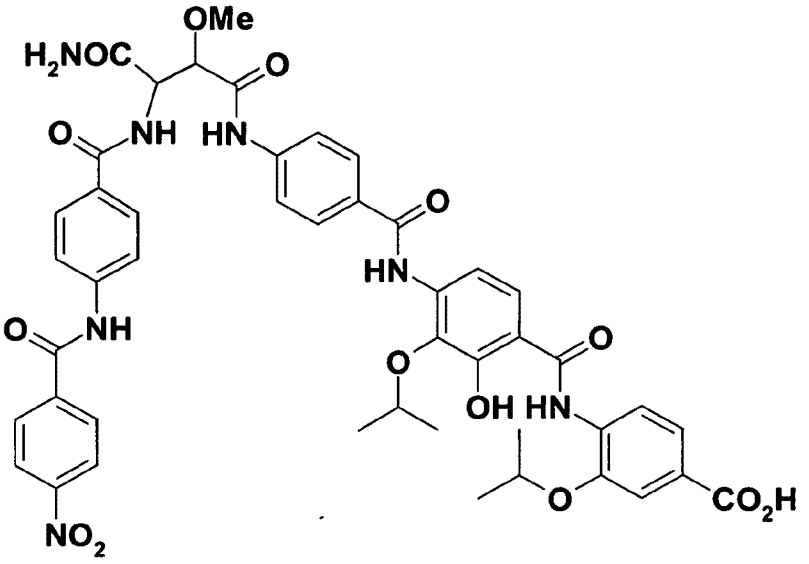


or

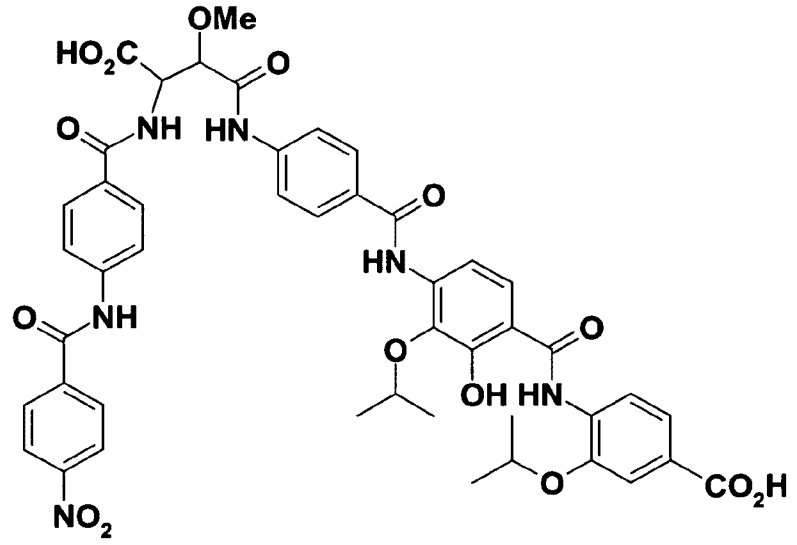


wherein R^9 is COOH or $CONH_2$ and R^{10} is COOH or $CONH_2$.

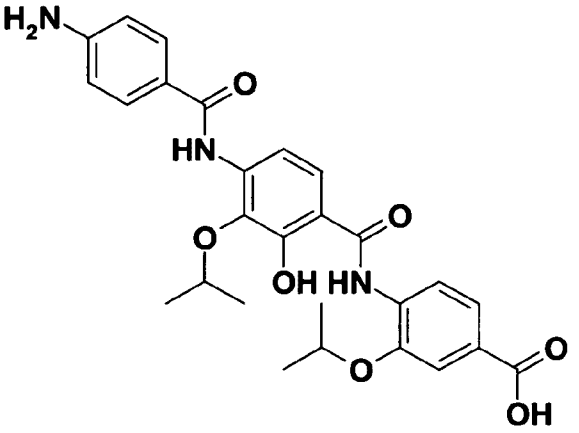
Especially preferred are the following compounds:



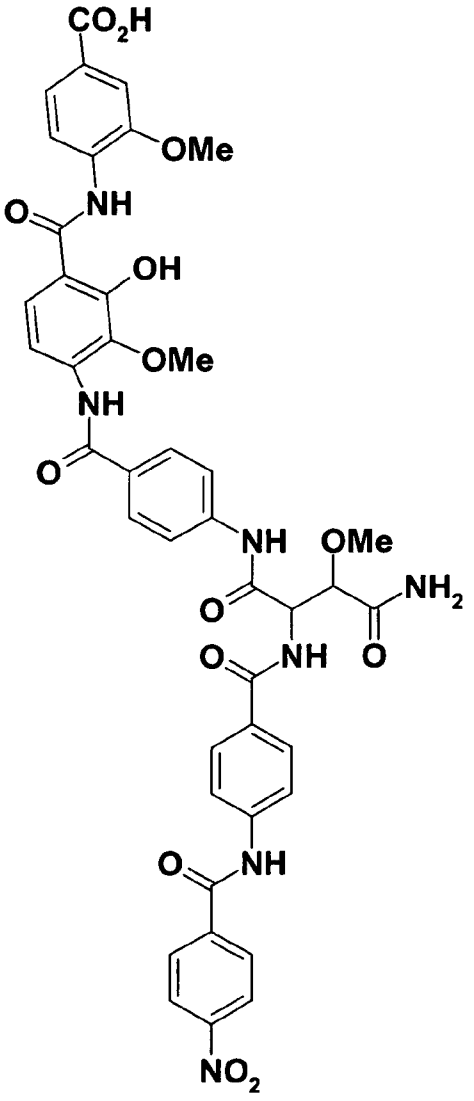
Cystobactamide A (**1**);



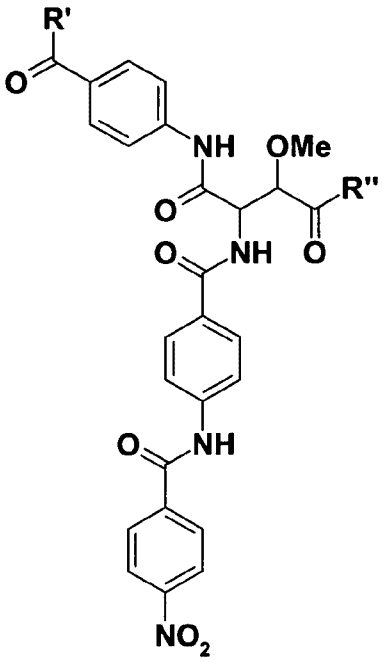
Cystobactamide B (**2**);



Cystobactamide C (**3**);

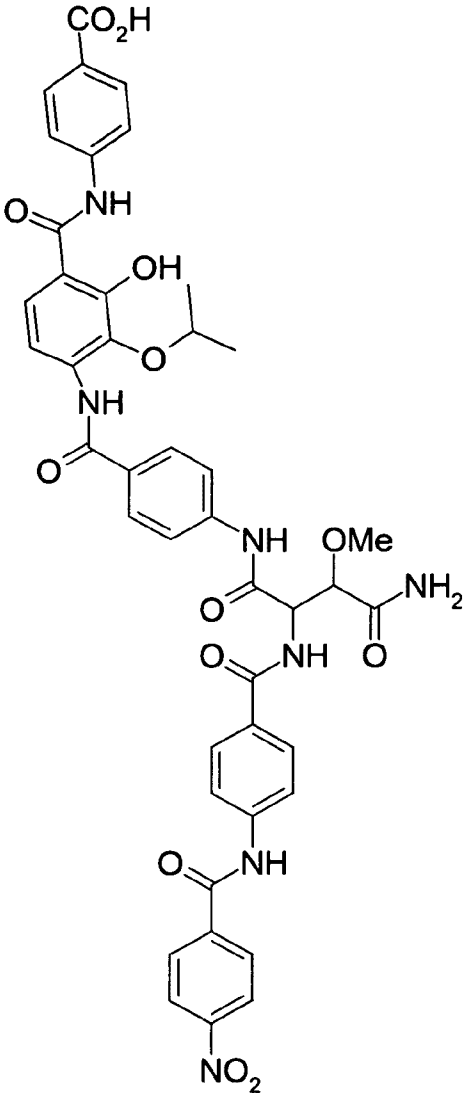


Cystobactamide D (**4**); and

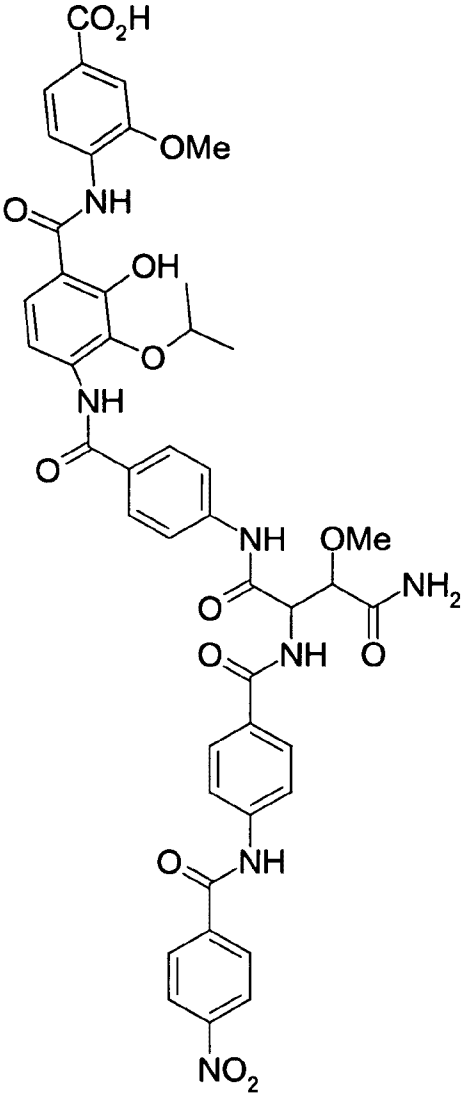


Cystobactamide E (**5**) (R' is NH_2 or OH and R" is NH_2 or OH).

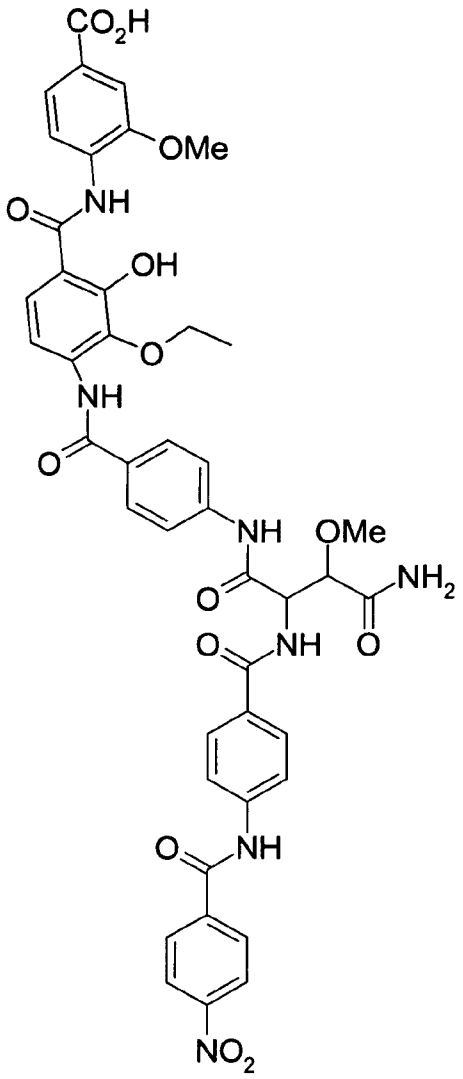
Moreover especially preferred are the following compounds:



Cystobactamide F (**6**),

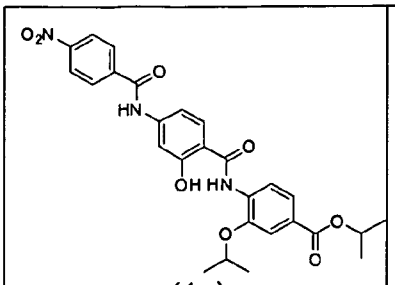


Cystobactamide G (**7**); and

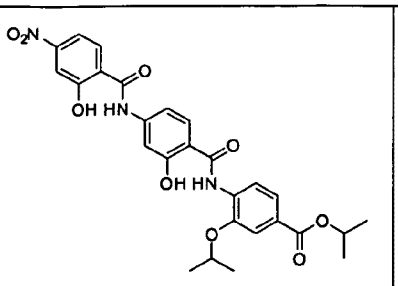


Cystobactamide H (**8**).

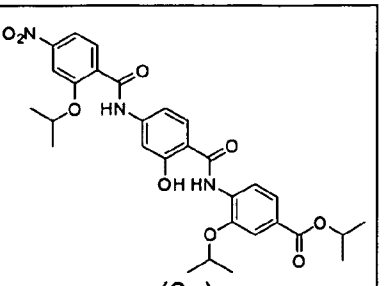
Moreover preferred are the following compounds:



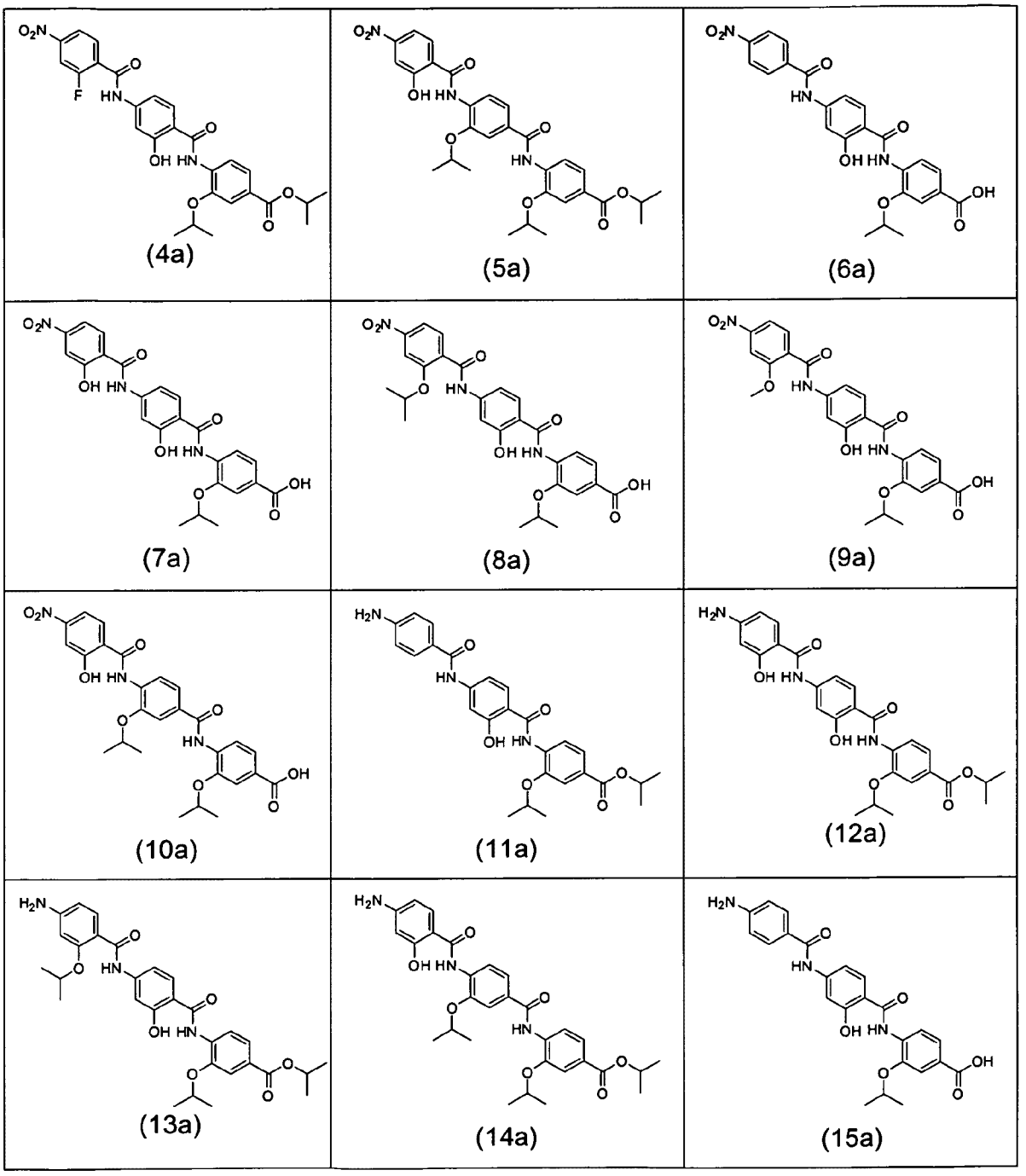
(1a)

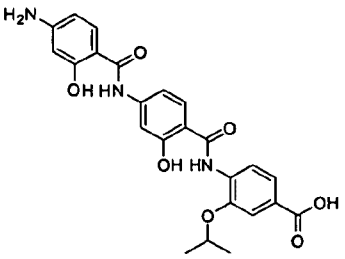


(2a)

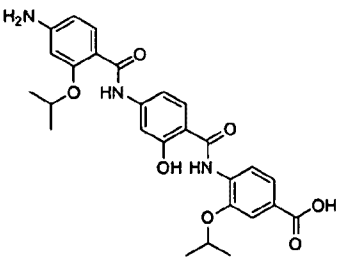


(3a)

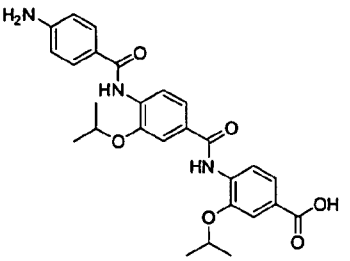

(4a)
(5a)
(6a)
(7a)
(8a)
(9a)
(10a)
(11a)
(12a)
(13a)
(14a)
(15a)



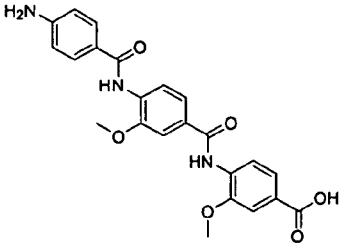
(16a)



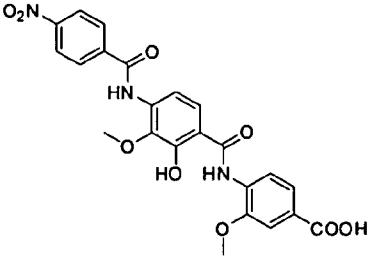
(17a)



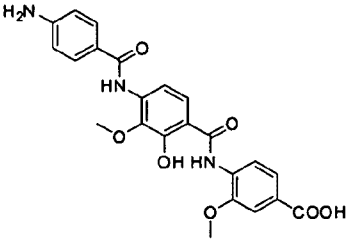
(18a)



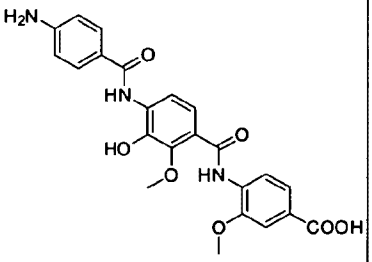
(19a)



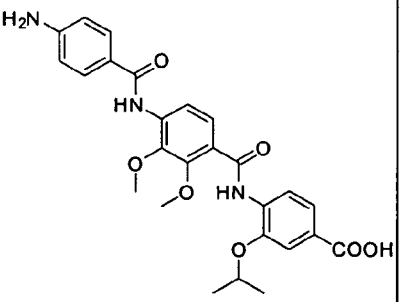
(20a)



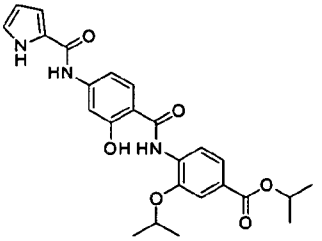
(21a)



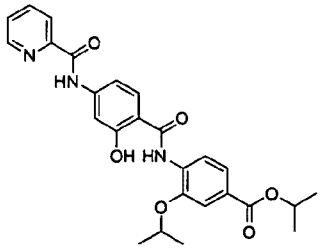
(22a)



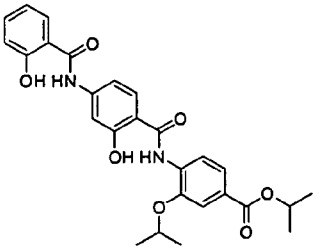
(23a)



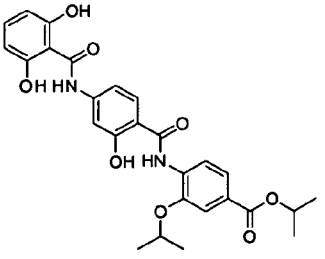
(24a)



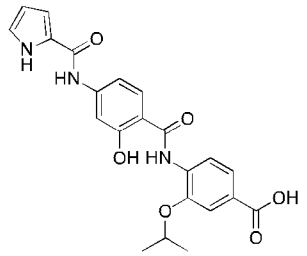
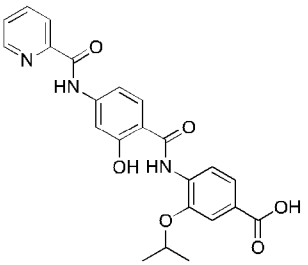
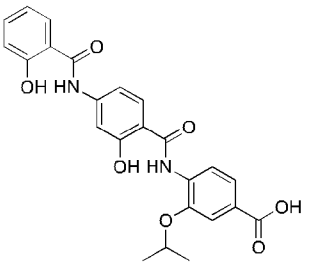
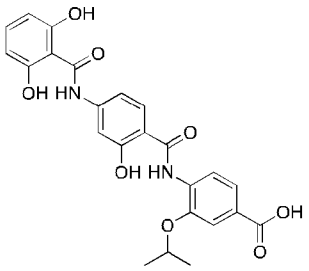
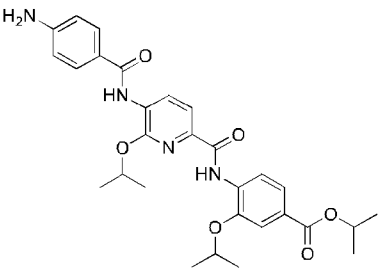
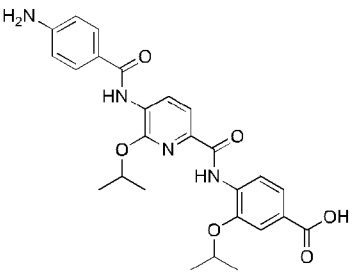
(25a)



(26a)



(27a)

 (28a)	 (29a)	 (30a)
 (31a)	 (32a)	 (33a)

According to one particular aspect, the invention relates to a compound of formula (I)

$$R^1\text{-}Ar^1\text{-}L^1\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

(I)

wherein

Ar^1 is an optionally substituted 1,4-phenylene group;

Ar^2 is an optionally substituted 1,4-phenylene group;

Ar^3 is an optionally substituted 1,4-phenylene group;

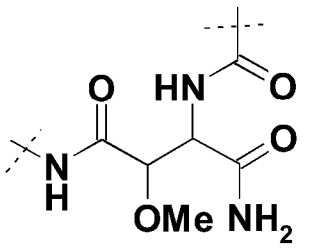
Ar^4 is an optionally substituted 1,4-phenylene group;

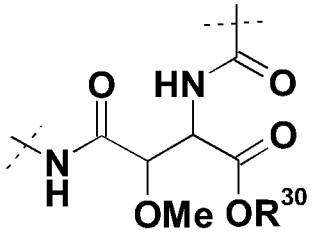
Ar^5 is an optionally substituted 1,4-phenylene group;

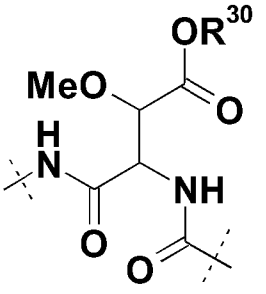
L^1 is NHCO (wherein the nitrogen atom is bound to Ar^1);

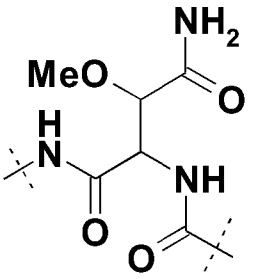
L^2 is NHCO (wherein the nitrogen atom is bound to Ar^2);

L^3 is a group of the following formula:



or


or


or


,

(wherein the NH group is bound to Ar^3), wherein R^{30} is a hydrogen atom or a C_{1-3}alkyl group (preferably, R^{30} is a hydrogen atom);

L^4 is absent or NHCO (wherein the nitrogen atom is bound to Ar^4);

R^1 is a group of formula $-NH_2$, $-NO_2$, $COOR^{11}$, or $-CONR^{12}R^{13}$; wherein R^{11}, R^{12} and R^{13} are independently a hydrogen atom or a C_{1-6} alkyl group (preferably, R^1 is a group of formula -COOH);

R^2 is a group of formula $-NH_2$, $-NO_2$, $COOR^{11a}$, or $-CONR^{12a}R^{13a}$; wherein R^{11a}, R^{12a} and R^{13a} are independently a hydrogen atom or a C_{1-6} alkyl group (preferably, R^2 is a group of formula $-NH_2$ or $-NO_2$);

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

Date Reçue/Date Received 2020-11-06

According to another particular aspect, the invention relates to the use of a compound as defined herein for the treatment or prophylaxis of bacterial infections.

The present invention further provides pharmaceutical compositions comprising one or more compounds described herein or a pharmaceutically acceptable salt, solvate or hydrate thereof, optionally in combination with one or more carrier substances and/or one or more adjuvants.

The present invention furthermore provides compounds or pharmaceutical compositions as described herein for use in the treatment and/or prophylaxis of bacterial infections, especially caused by *E. coli*, *P. aeruginosa, A. baumannii,* other Gram-negative bacteria, and Gram-positive bacteria.

Moreover preferably, the present invention provides compounds for use in the treatment and/or prophylaxis of bacterial infections, especially caused by *Pseudomonas aeruginosa* and other Gram-negative bacteria.

It is a further object of the present invention to provide a compound as described herein or a pharmaceutical composition as defined herein for the preparation of a medicament for the treatment and/or prophylaxis of bacterial infections, especially caused by selected Gram-negative bacteria and Gram-positive bacteria.

Date Reçue/Date Received 2020-11-06

Examples of pharmacologically acceptable salts of sufficiently basic compounds are salts of physiologically acceptable mineral acids like hydrochloric, hydrobromic, sulfuric and phosphoric acid; or salts of organic acids like methanesulfonic, p-toluenesulfonic, lactic, acetic, trifluoroacetic, citric, succinic, fumaric, maleic and salicylic acid. Further, a sufficiently acidic compound may form alkali or earth alkali metal salts, for example sodium, potassium, lithium, calcium or magnesium salts; ammonium salts; or organic base salts, for example methylamine, dimethylamine, trimethylamine, triethylamine, ethylenediamine, ethanolamine, choline hydroxide, meglumin, piperidine, morpholine, tris-(2-hydroxyethyl)amine, lysine or arginine salts; all of which are also further examples of salts of the compounds described herein. The compounds described herein may be solvated, especially hydrated. The hydratization/hydration may occur during the process of production or as a consequence of the hygroscopic nature of the initially water free compounds. The solvates and/or hydrates may e.g. be present in solid or liquid form.

The therapeutic use of the compounds described herein, their pharmacologically acceptable salts, solvates and hydrates, respectively, as well as formulations and pharmaceutical compositions also lie within the scope of the present invention.

The pharmaceutical compositions according to the present invention comprise at least one compound described herein and, optionally, one or more carrier substances and/or adjuvants.

As mentioned above, therapeutically useful agents that contain compounds described herein, their solvates, salts or formulations are also comprised in the scope of the present invention. In general, the compounds described herein will be administered by using the known and acceptable modes known in the art, either alone or in combination with any other therapeutic agent.

For oral administration such therapeutically useful agents can be administered by one of the following routes: oral, e.g. as tablets, dragees, coated tablets, pills,

semisolids, soft or hard capsules, for example soft and hard gelatine capsules, aqueous or oily solutions, emulsions, suspensions or syrups, parenteral including intravenous, intramuscular and subcutaneous injection, e.g. as an injectable solution or suspension, rectal as suppositories, by inhalation or insufflation, e.g. as a powder formulation, as microcrystals or as a spray (e.g. liquid aerosol), transdermal, for example via an transdermal delivery system (TDS) such as a plaster containing the active ingredient or intranasal. For the production of such tablets, pills, semisolids, coated tablets, dragees and hard, e.g. gelatine, capsules the therapeutically useful product may be mixed with pharmaceutically inert, inorganic or organic excipients as are e.g. lactose, sucrose, glucose, gelatine, malt, silica gel, starch or derivatives thereof, talc, stearinic acid or their salts, dried skim milk, and the like. For the production of soft capsules one may use excipients as are e.g. vegetable, petroleum, animal or synthetic oils, wax, fat, and polyols. For the production of liquid solutions, emulsions or suspensions or syrups one may use as excipients e.g. water, alcohols, aqueous saline, aqueous dextrose, polyols, glycerin, lipids, phospholipids, cyclodextrins, vegetable, petroleum, animal or synthetic oils. Especially preferred are lipids and more preferred are phospholipids (preferred of natural origin; especially preferred with a particle size between 300 to 350 nm) preferred in phosphate buffered saline (pH = 7 to 8, preferred 7.4). For suppositories one may use excipients as are e.g. vegetable, petroleum, animal or synthetic oils, wax, fat and polyols. For aerosol formulations one may use compressed gases suitable for this purpose, as are e.g. oxygen, nitrogen and carbon dioxide. The pharmaceutically useful agents may also contain additives for conservation, stabilization, e.g. UV stabilizers, emulsifiers, sweetener, aromatizers, salts to change the osmotic pressure, buffers, coating additives and antioxidants.

In general, in the case of oral or parenteral administration to adult humans weighing approximately 80 kg, a daily dosage of about 1 mg to about 10,000 mg, preferably from about 5 mg to about 1,000 mg, should be appropriate, although the upper limit may be exceeded when indicated. The daily dosage can be administered as a single dose or in divided doses, or for parenteral administration, it may be given as continuous infusion or subcutaneous injection.

The compounds of the present invention can be prepared by fermentation (e.g. by fermentation of strain MCy8071 DSM27004) or by chemical synthesis applying procedures known to a person skilled in the art.

For example the compounds of the present invention can be prepared according to the following procedures:

Starting from the respective optionally substituted building blocks (e.g. Ar^1, Ar^2, Ar^3, Ar^4 and Ar^5), these building blocks can be linked to each other using acid chlorides or coupling reagents which are known to a person skilled in the art, e.g. according to the following reaction scheme:

$$R^1\text{-}Ar^1\text{-}NH_2 + HOOC\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

$$R^1\text{-}Ar^1\text{-}NH_2 + HO_3SC\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

If L^1, L^2, L^3 and/or L^4 is a group of formula -CH=CH- (or another olefine group), the respective optionally substituted building blocks (e.g. Ar^1, Ar^2, Ar^3, Ar^4 and Ar^5) can be linked to each other using a Wittig or a Horner reaction, e.g. according to the following reaction scheme:

$$R^1\text{-}Ar^1\text{-}CHO + BrPh_3P\text{-}CH_2\text{-}\ Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

$$R^1\text{-}Ar^1\text{-}CHO + (EtO)_2OPCH_2\text{-}\ Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

If L^1, L^2, L^3 and/or L^4 is a heterocycloalkyl or a heteroaryl group, the respective optionally substituted building blocks (e.g. Ar^1, Ar^2, Ar^3, Ar^4 and Ar^5) can be linked to each other applying similar reaction conditions.

Identification of the cystobactamide biosynthesis gene cluster:

The genome of the cystobactamid producer has been sequenced by shotgun-sequencing. As the main building block of the cystobactamides is the non-proteinogenic amino acid p-aminobenzoic acid (PABA), p-aminobenzoic acid synthase (query, NP_415614) was used as query for the identification of a putative cystobactamide biosynthetic cluster in the genome of Cbv34. Importantly, a p-aminobenzoic acid synthase homologue could be identified (CysD, figure 12 and table A), which is forming an operon with non-ribosomal peptide synthases (CysG, H and K) in the context of an *in silico* predicted ~48kb large NRPS cluster (figure 12, assignment: table A). The genes in this NRPS cluster have been analysed by pfam, NCBI BLAST and phyre2. Aside the p-aminobenzoic acid synthase homologue, two further PABA biosynthetic enzymes can be found in the cluster: an aminodeoxychorismate lyase (CysI) and a 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP) synthase (CysN). DAHP synthase (CysN) is a key enzyme for the production of shikimate and chorismate. In the main trunk of the shikimate pathway, D-erythrose 4-phosphate and phosphoenolpyruvate (DAHP synthase) are converted via shikimate to chorismate. CysI and CysD allow the direct biosynthesis of PABA from chorismate. Furthermore, the cluster contains a p-aminobenzoic acid N-oxygenase homologue (CysR).

Figure 12 shows the cystobactamide biosynthetic cluster of the invention.

A recombinant biosynthesis cluster capable of synthesizing a cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and H, wherein the cluster comprises all of the polypeptides, or a functional variant thereof, according to SEQ ID NOs. 40 to 73.

The term "functional variant" as used herein denotes a polypeptide having a sequence that is at least 85%, 90%, 95% or 99% identical to a polypeptide sequence described herein. A "functional variant" of a polypeptide may retain amino acids residues recognized as conserved for the polypeptide in nature, and/or may have non-conserved amino acid residues. Amino acids can be, relative to the native polypeptide, substituted (different), inserted, or deleted, but the variant has generally

similar (enzymatic) activity or function as compared to a polypeptide described herein. A "functional variant" may be found in nature or be an engineered mutant (recombinant) thereof.

The term "identity" refers to a property of sequences that measures their similarity or relationship. Identity is measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100.

The terms "protein", "polypeptide", "peptide" as used herein define an organic compound made of two or more amino acid residues arranged in a linear chain, wherein the individual amino acids in the organic compound are linked by peptide bonds, i.e. an amide bond formed between adjacent amino acid residues. By convention, the primary structure of a protein is reported starting from the amino-terminal (N) end to the carboxyl-terminal (C) end.

As used herein, "comprising", "including", "containing", "characterized by", and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising", etc. is to be interpreted as including the more restrictive term "consisting of".

As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim.

When trade names are used herein, it is intended to independently include the trade name product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product.

In general, unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with general textbooks and dictionaries.

Preferably, the NRPS enzyme of the invention is a not naturally occurring NRPS. The NRPS of the invention may also be a hybrid NRPS comprising modules, domains, and/or portions thereof, or functional variants thereof, from two or more NRPSs or from one or more polyketide synthase(s) (PKSs).

The cystobactamide biosynthesis cluster of the invention preferably includes the elements of Table A.

Table A: Cystobactamide gene cluster of the invention. Gene and NRPS domain annotation with the gene cluster sequence corresponding to SEQ ID NO. 1.

location within the gene cluster sequence (bp)						NRPS						
						Domains	location within the gene cluster sequence (bp)			location within the protein sequence (aa)		
Name	Min.	Max.	direction	Length	aa		length	Min.	Max.	length	Min.	Max.
Orf1	15	845	reverse	831	276							
Orf2	912	1148	reverse	237	78							
Orf3	1339	1827	reverse	489	162							
Orf4	1907	2170	reverse	264	87							
Orf5	2347	2796	reverse	450	149							
CysT	3035	6838	reverse	3804	1267							
CysS	7049	8977	reverse	1929	642							
CysR	9086	10087	reverse	1002	333							
CysQ	10162	10956	reverse	795	264							
CysP	11029	11730	reverse	702	233							
CysO	11764	12375	reverse	612	203							
CysA	12715	12927	forward	213	70							
CysB	12996	13949	forward	954	317							
CysC	13959	15338	forward	138	45							
CysD	15464	17662	forward	2199	732							
CysE	17749	18480	forward	732	243							
CysF	18503	19540	forward	1038	345							
CysG	19580	25558	forward	5979	1992	AMP-binding domain	1451	19694	21145	483	39	521
						PCP domain	209	21221	21430	69	548	616
						Condensation_LCL domain	893	21485	22378	297	636	932
						AMP-binding domain	1451	22880	24331	483	1101	1583

						PCP domain	215	24404	24619	71	1609	1679
						Thioesterase domain	788	24728	25516	262	1717	1978
CysH	25626	28553	forward	2928	975	AMP-binding domain	1199	25737	26936	399	38	436
						novel domain type	332	27231	27563	110	536	645
						AMP binding domain C-terminus	170	28032	28202	56	803	858
						PCP domain	197	28284	28481	65	887	951
CysI	28555	29373	forward	819	272							
CysJ	29392	30375	forward	984	327							
CysK	30450	44087	forward	13638	4545	Condensation_LCL domain	323	30459	30782	107	4	110
						AMP-binding domain	1505	31239	32744	501	264	764
						PCP domain	197	32820	33017	65	791	855
						Condensation_LCL domain	893	33072	33965	297	875	1171
						AMP-binding domain	1505	34461	35966	501	1338	1838
						PCP domain	197	36042	36239	65	1865	1929
						Condensation_LCL domain	890	36285	37175	296	1946	2241
						AMP-binding domain	1574	37668	39242	524	2407	2930
						PCP domain	359	39165	39524	119	2906	3024
						Condensation_LCL domain	893	39579	40472	297	3044	3340
						AMP-binding domain	1505	40968	42473	501	3507	4007
						PCP domain	197	42549	42746	65	4034	4098
						Condensation_LCL domain	896	42801	43697	298	4118	4415
CysL	44084	47155	forward	3072	1023	AMP-binding domain	1445	45665	47110	481	528	1008
CysM	47152	47268	forward	117	38							
CysN	47280	48353	forward	1074	357							
Orf6	48490	50067	reverse	1578	525							
Orf7	50064	50849	reverse	786	261							
Orf8	50855	52156	reverse	1302	433							
Orf9	52161	54266	reverse	2106	701							
Orf10	54266	55027	reverse	762	253							
Orf11	55486	56679	forward	1194	397							
Orf12	56760	57134	forward	375	124							
Orf13	57166	57504	reverse	339	112							
Orf14	57504	58418	reverse	915	304							

The present invention also provides isolated, synthetic or recombinant nucleic acids that encode NRPSs of the invention. Said nucleic acids include nucleic acids that

include a portion or all of a NRPS of the invention, nucleic acids that further include regulatory sequences, such as promoter and translation initiation and termination sequences, and can further include sequences that facilitate stable maintenance in a host cell, i.e., sequences that provide the function of an origin of replication or facilitate integration into host cell chromosomal or other DNA by homologous recombination. These NRPSs may be used as research tools or as modules in recombinant NRPS or PKS clusters.

Preferably, the invention relates to an isolated, synthetic or recombinant nucleic acid comprising:

(i) a sequence encoding a cystobactamide biosynthesis cluster, wherein the sequence has a sequence identity to the full-length sequence of SEQ ID NO. 1 from at least 85%, 90%, 95%, 96%, 97%, 98%, 98.5%, 99%, or 99.5% to 100%;

(ii) a sequence encoding a NRPS, wherein the sequence has a sequence identity to the full-length sequence of any of SEQ ID NOs. 8, 9, 12 or 13 from at least 85%, 90%, 95%, 96%, 97%, 98%, 98.5%, 99%, or 99.5% to 100%;

(iii) a sequence completely complementary to the full length sequence of any nucleic acid sequence of (i) or (ii); or

(iv) a sequence encoding a polypeptide according to any of SEQ ID NOs. 46, 47, 50 or 51.

The phrases "nucleic acid" or "nucleic acid sequence" as used herein refer to an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, natural or synthetic in origin. "Oligonucleotide" includes either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands that may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide can ligate to a fragment that has not been dephosphorylated. A "coding sequence" of or a "nucleotide sequence encoding" a particular polypeptide or protein, is a nucleic acid sequence which is transcribed and

translated into a polypeptide or protein when placed under the control of appropriate regulatory sequences. The nucleic acids used to practice this invention may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993). A nucleic acid encoding a polypeptide of the invention is assembled in appropriate phase with a leader sequence capable of directing secretion of the translated polypeptide or fragment thereof.

The term "isolated" as used herein means that the material, e.g., a nucleic acid, a polypeptide, a vector, a cell, is removed from its original environment, e.g., the natural environment if it is naturally occurring. For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition and still be isolated in that such vector or composition is not part of its natural environment.

The term "synthetic" as used herein means that the material, e.g. a nucleic acid, has been synthesized in vitro by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22: 1859.

The term "recombinant" means that the nucleic acid is adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. Backbone molecules according to the invention include nucleic acids such as cloning and expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. Recombinant polypeptides of the invention, generated from these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including bacterial, mammalian, yeast, insect or plant cell expression systems.

Also provided is a vector comprising at least one nucleic acid according to the invention. The vector may be a cloning vector, an expression vector or an artificial chromosome.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors, including cloning and expression vectors, comprise a nucleic acid of the invention or a functional equivalent thereof. Nucleoc acids of the invention can be incorporated into a recombinant replicable vector, for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, the invention also provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below. The vector into which the expression cassette or nucleic acid of the invention is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of the vector will often depend on the host cell into which it is to be introduced. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, N. Y., (1989).

A vector according to the invention may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e. g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication, and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms "plasmid" and "vector" can be used interchangeably herein as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as cosmid, viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses) and phage vectors which serve equivalent functions.

Vectors according to the invention may be used in vitro, for example for the production of RNA or used to transfect or transform a host cell.

A vector of the invention may comprise two or more, for example three, four or five, nucleic acids of the invention, for example for overexpression.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which

means that the recombinant expression vector includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operationally linked to the nucleic acid sequence to be expressed.

Within a vector, such as an expression vector, "operationally linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell), i.e. the term "operationally linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence such as a promoter, enhancer or other expression regulation signal "operationally linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences or the sequences are arranged so that they function in concert for their intended purpose, for example transcription initiates at a promoter and proceeds through the DNA sequence encoding the polypeptide.

The term "regulatory sequence" or "control sequence" is intended to include promoters, operators, enhancers, attenuators and other expression control elements (e.g., polyadenylation signal). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

The term regulatory or control sequences includes those sequences which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in a certain host cell (e.g. tissue-specific regulatory sequences).

A vector or expression construct for a given host cell may thus comprise the following elements operationally linked to each other in a consecutive order from the 5'-end to 3'-end relative to the coding strand of the sequence encoding the polypeptide of the invention: (i) a promoter sequence capable of directing transcription of the nucleotide

sequence encoding the polypeptide in the given host cell; (ii) optionally, a signal sequence capable of directing secretion of the polypeptide from the given host cell into a culture medium; (iii) optionally, a sequence encoding for a C-terminal, N-terminal or internal epitope tag sequence or a combination of the aforementioned allowing purification, detection or labeling of the polypeptide; (iv) a nucleic acid sequence of the invention encoding a polypeptide of the invention; and preferably also (v) a transcription termination region (terminator) capable of terminating transcription downstream of the nucleotide sequence encoding the polypeptide. Particular named bacterial promoters include lad, lacZ, T3, T7, SP6, K1F, tac, tet, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. Downstream of the nucleotide sequence according to the invention there may be a 3' untranslated region containing one or more transcription termination sites (e. g. a terminator). The origin of the terminator is less critical. The terminator can, for example, be native to the DNA sequence encoding the polypeptide. Preferably, the terminator is endogenous to the host cell (in which the nucleotide sequence encoding the polypeptide is to be expressed). In the transcribed region, a ribosome binding site for translation may be present. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG (or TUG or GUG in prokaryotes) at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

Enhanced expression of a polynucleotide of the invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and/or terminator regions, which may serve to increase expression and, if desired, secretion levels of the protein of interest from the expression host and/or to provide for the inducible control of the expression of a polypeptide of the invention. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The vectors, such as expression vectors, of the

invention can be introduced into host cells to thereby produce proteins or peptides, encoded by nucleic acids as described herein.

The vectors, such as recombinant expression vectors, of the invention can be designed for expression of a portion or all of a NRPS of the invention in prokaryotic or eukaryotic cells. For example, a portion or all of a NRPS of the invention can be expressed in bacterial cells such as E. coli, Bacillus strains, insect cells (using baculovirus expression vectors), filamentous fungi, yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Representative examples of appropriate hosts are described hereafter. Appropriate culture media and conditions for the above-described host cells are known in the art.

As set out above, the term "control sequences" or "regulatory sequences" is defined herein to include at least any component which may be necessary and/or advantageous for the expression of a polypeptide. Any control sequence may be native or foreign to the nucleic acid sequence of the invention encoding a polypeptide. Such control sequences may include, but are not limited to, a promoter, a leader, optimal translation initiation sequences (as described in Kozak, 1991 , J. Biol. Chem. 266:19867- 19870), a secretion signal sequence, a pro-peptide sequence, a polyadenylation sequence, a transcription terminator. At a minimum, the control sequences typically include a promoter, and transcriptional and translational stop signals. A stably transformed microorganism is one that has had one or more DNA fragments introduced such that the introduced molecules are maintained, replicated and segregated in a growing culture. Stable transformation may be due to multiple or single chromosomal integration(s) or by (an) extrachromosomal element(s) such as (a) plasmid vector(s). A plasmid vector is capable of directing the expression of polypeptides encoded by particular DNA fragments. Expression may be constitutive or regulated by inducible (or repressible) promoters that enable high levels of transcription of functionally associated DNA fragments encoding specific polypeptides.

Expression vectors of the invention may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed, e.g., genes which render the bacteria resistant to drugs such as chloramphenicol, erythromycin, kanamycin, neomycin, tetracycline, as well as ampicillin and other penicillin derivatives like carbenicillin. Selectable markers can also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

The appropriate polynucleotide sequence may be inserted into the vector by a variety of procedures. In general, the polynucleotide sequence is ligated to the desired position in the vector following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, blunt ends in both the insert and the vector may be ligated. A variety of cloning techniques are disclosed in Ausubel et al. Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. 1997 and Sambrook et al, Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press (1989). The polynucleotide sequence may also be cloned using homologous recombination techniques including in vitro as well as in vivo recombination. Such procedures and others are deemed to be within the scope of those skilled in the art. The vector may be, for example, in the form of a plasmid, a viral particle, or a phage. Other vectors include chromosomal, nonchromosomal and synthetic polynucleotide sequences, derivatives of SV40; bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and bacteriophage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus and pseudorabies.

The invention also provides an engineered or recombinant host cell, i.e. a transformed cell comprising a nucleic acid sequence of the invention as a heterologous or non-native polynucleotide, e.g. a sequence encoding the cystobactamide biosynthesis cluster or a NRPS of the invention, or a vector of the invention. The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells, eukaryotic cells, such as bacterial cells, fungal cells, yeast cells, mammalian cells, insect cells, or plant cells.

Preferred mammalian cells include e.g. Chinese hamster ovary (CHO) cells, COS cells, 293 cells, PerC6 cells, hybridomas, Bowes melanoma or any mouse or any human cell line. Exemplary insect cells include any species of *Spodoptera* or *Drosophila*, including *Drosophila* S2 and *Spodoptera* Sf-9. Exemplary fungal cells include any species of *Aspergillus*. Preferred yeast cell include, e. g. a cell from a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain. More preferably from *Kluyveromyces lactis*, *S. cerevisiae*, *Hansenula polymorpha*, *Yarrowia lipolytica*, or *Pichia pastoris*. According to the invention, the host cell may be a prokaryotic cell. Preferably, the prokaryotic host cell is a bacterial cell. The term "bacterial cell" includes both Gram-negative and Gram-positive as well as archaeal microorganisms. Suitable bacteria may be selected from e.g. *Escherichia*, *Anabaena*, *Caulobacter*, *Gluconobacter*, *Rhodobacter*, *Pseudomonas*, *Paracoccus*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Rhizobium* (*Sinorhizobium*), *Flavobacterium*, *Klebsiella*, *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Staphylococcus* or *Streptomyces*. Preferably, the bacterial cell is selected from the group consisting of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. puntis*, B. megaterium, *B. halodurans*, *B. pumilus*, *G. oxydans*, *Caulobacter crescentus* CB 15, *Methylobacterium extorquens*, *Rhodobacter sphaeroides*, *Pseudomonas putida*, *Paracoccus zeaxanthinifaciens*, *Paracoccus denitrificans*, *E. coli*, *C. glutamicum*, *Staphylococcus carnosus*, *Streptomyces lividans*, *Sinorhizobium melioti* and *Rhizobium radiobacter*. The selection of an appropriate host is within the abilities of those skilled in the art.

The vector can be introduced into the host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)). The nucleic acids or vectors of the invention may be introduced into the cells for screening, thus, the nucleic acids enter the cells in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type.

Exemplary methods include $CaPO_4$ precipitation, liposome fusion, lipofection (e.g., LIPOFECTIN™), electroporation, viral infection, etc. The candidate nucleic acids may stably integrate into the genome of the host cell (for example, with retroviral introduction) or may exist either transiently or stably in the cytoplasm (i.e. through the use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.). As many pharmaceutically important screens require human or model mammalian cell targets, retroviral vectors capable of transfecting such targets can be used.

Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the nucleic acids of the invention. Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow them to produce the desired polypeptide or fragment thereof. Cells can be harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps. The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Depending upon the host employed in a recombinant production procedure, the polypeptides produced by host cells containing the vector may be glycosylated or may be non-glycosylated. Polypeptides of the invention may

or may not also include an initial methionine amino acid residue. Cell-free translation systems can also be employed to produce a polypeptide of the invention. Cell-free translation systems can use mRNAs transcribed from a DNA construct comprising a promoter operationally linked to a nucleic acid encoding the polypeptide or fragment thereof. In some aspects, the DNA construct may be linearized prior to conducting an in vitro transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

Host cells containing the polynucleotides of interest, e.g., nucleic acids of the invention, can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions such as temperature, pH and the like, are those previously used with the host cell selected for expression and will be apparent to the ordinarily skilled artisan. The clones which are identified as having the specified enzyme activity may then be sequenced to identify the polynucleotide sequence encoding a portion or all of a NRPS of the invention.

Recombinant DNA can be introduced into the host cell by any means, including, but not limited to, plasmids, cosmids, phages, yeast artificial chromosomes or other vectors that mediate transfer of genetic elements into a host cell. These vectors can include an origin of replication, along with *cis*-acting control elements that control replication of the vector and the genetic elements carried by the vector. Selectable markers can be present on the vector to aid in the identification of host cells into which genetic elements have been introduced. Means for introducing genetic elements into a host cell (e.g. cloning) are well known to the skilled artisan. Other cloning methods include, but are not limited to, direct integration of the genetic material into the chromosome. This can occur by a variety of means, including cloning the genetic elements described herein on non-replicating plasmids flanked by homologous DNA sequences of the host chromosome; upon transforming said recombinant plasmid into a host the genetic elements can be introduced into the chromosome by DNA recombination. Such recombinant strains can be recovered if

the integrating DNA fragments contain a selectable marker, such as antibiotic resistance. Alternatively, the genetic elements can be directly introduced into the chromosome of a host cell without use of a non-replicating plasmid. This can be done by synthetically producing DNA fragments of the genetic elements in accordance to the present invention that also contain homologous DNA sequences of the host chromosome. Again if these synthetic DNA fragments also contain a selectable marker, the genetic elements can be inserted into the host chromosome.

The cystobactamide biosynthesis cluster or a NRPS of the invention may be favorably expressed in any of the above host cells. Thus, the present invention provides a wide variety of host cells comprising one or more of the isolated, synthetic or recombinant nucleic acids and/or NRPSs of the present invention. The host cell, when cultured under suitable conditions, is capable of producing a cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and H that it otherwise does not produce, or produces at a lower level, in the absence of a nucleic acid of the invention.

The invention also relates to an isolated, synthetic or recombinant polypeptide having an amino acid sequence according to any of SEQ ID NOs. 40 to 73, or an amino acid sequence encoded by a nucleic acid of the invention.

The present invention further provides a method for the preparation of a cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and H, said method generally comprising: providing a host cell of the present invention, and culturing said host cell in a suitable culture medium under suitable conditions such that at least one cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and His produced. The method may further comprise a step of isolating a cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and H, i.e. separating and retaining the compound from the culture broth. The isolation step may be carried out using affinity chromatography, anion exchange chromatography, or reversed phase chromatography.

Examples

Conditions of production

Strain for production

The strain *Cystobacter velatus* MCy8071 belongs to the order Myxococcales (Myxobacteria), suborder Cystobacterineae, family Cystobacteraceae, genus Cystobacter. The comparison of the partial 16S rRNA gene sequences with sequences of a public database (BLAST, Basic Local Alignment Search Tool provided by NCBI, National Center for Biotechnology Information) revealed 100 % similarity to *Cystobacter velatus* strain DSM 14718.

MCy8071 was isolated at the Helmholtz Centre for Infection Research (HZI, formerly GBF) from a Chinese soil sample collected in 1982. The strain was deposited at the German Collection of Microorganisms in Braunschweig (DSM) in March 2013 under the designation DSM 27004.

Cultivation

The strain MCy8071 grows well on yeast-agar (VY/2: 0.5 % *Saccharomyces cerevisiae*, 0.14 % $CaCl_2$ x 2 H_2O, 0.5 µg vitamine B_{12}/l, 1.5 % agar, pH 7.4), CY-agar (casitone 0.3 %, yeast extract 0.1 %, $CaCl_2$ x 2 H_2O 0.1%, agar 1.5 %, pH 7.2) and P-agar (peptone Marcor 0.2 %, starch 0.8 %, single cell protein probione 0.4 %, yeast extract 0.2 %, $CaCl_2$ x 2 H_2O 0.1 %, $MgSO_4$ 0.1 %, Fe-EDTA 8mg/l, 1.5 % agar, pH 7.5). The working culture was nurtured in liquid medium CY/H (50 % CY-medium + 50 mM Hepes, 50 % H-medium: soy flour 0.2 %, glucose 0.8 %, starch 0.2 %, yeast extract 0.2 %, $CaCl_2$ x 2 H_2O 0.1 %, $MgSO_4$ 0.1 %, Fe-EDTA 8mg/l, Hepes 50 mM pH 7.4). Liquid cultures were shaken at 180 rpm at 30 °C. For conservation aliquots à 2 ml of a three days old culture were stored at –80 °C. Reactivation, even after several years, is no problem on the above mentioned agar plates or in 20 ml CY/H-medium (in 100 ml Erlenmeyer flasks with plugs and aluminium-cap). After one-two days the 20 ml cultures can be upscaled to 100 ml.

Morphological description

After two days in liquid medium CY/H the rod-shaped cells of strain MCy8071 have a length of 9.0 – 14.5 µm and width of 0.8 – 1.0 µm. On the above mentioned agar-plates swarming is circular. On VY/2-agar the swarm is thin and transparent. Yeast degradation is visible on VY/2-agar. On CY-agar the culture looks transparent-orange. On P-agar cell mass production is distinctive and swarming behaviour is reduced. The colony colour is orange-brown. Starch in P-agar is degraded.

MCy8071 is resistant against the following antibiotics: ampicillin, gentamycin, hygromycin, polymycin, bacitracin, spectinomycin, neomycin, and fusidinic acid. Weak growth is possible with cephalosporin and kasugamycin and no growth is possible with thiostrepton, trimethoprin, kanamycin, and oxytetracycline (final concentration of all antibiotics was adjusted to 50 µg ml^{-1}).

Production of Cystobactamides A, B, C, D, E, F, G and H

The strain produces in complex media. He prefers nitrogen containing nutrients like single cell protein (Probion) and products of protein decomposition like peptone, tryptone, yeast extract, soy flour and meat extract. Here the production is better with several of the mentioned proteinmixtures compared to a single one.

Cystobactamides are produced within the logarithmical to the stationary phase of growth. After two days in 100 liter fermentation (medium E) the amount of products did not increase anymore.

Cystobactamides are delivered to the medium and bind to XAD-adsorber resin. XAD is sieved by a metal sieve and eluted in acetone. Different production temperatures were tested (21 °C, 30 °C, 37 °C and 42 °C) whereby at 42 °C no production was possible. The optimal temperature was at 30 °C with maximal aeration.

Fermentation of MCy8071 was conducted in a 150 liter fermenter with 100 liter medium E (skimmed milk 0.4 %, soy flour 0.4 %, yeast extract 0.2 %, starch 1.0 %,

$MgSO_4$ 0.1 %, Fe-EDTA 8mg/l, glycerine 0.5 %; pH 7.4) and in a 100 liter fermenter with 70 liter medium M (soy-peptone 1.0 %, maltose 1.0 %, $CaCl_2$ x 2 H_2O 0.1 %, $MgSO_4$ 0.1 %, Fe-EDTA 8mg/l; pH 7.2) for four days at 30 °C. The pH was regulated with potassium hydroxide (2.5 %) and sulfuric acid between 7.2 and 7.4. The stirrer speed was 100 – 400 rpm, aerated with 0,05 vvm compressed air. The dissolved oxygen content within the fermentation broth was regulated by the stirrer speed to pO_2 40 %. To bind cystobactamides 1 % adsorber resin was added to the fermentation broth. The fermenter was inoculated with 5 liter of a three days old pre-culture (E or M-medium, respectively). The production during the fermentation process was checked by HPLC-MS-analyses and serial dilution test of the methanol extract against *Escherichia coli*. The strain produces Cystobactamides A, B, C, D, E, F, G and H.

Knock-out Experiments

To confirm that the cystobactamide biosynthesis gene cluster is responsible for the production of the cystobactamides, a knock-out (KO) experiment was carried out, where CysK (NRPS) and CysL (benzoyl-CoA ligase) was knocked out, respectively. Specifically, PCR products of 1000bp fragments of CysK and CysL genes were produced from MCy8071 genomic DNA using Taq polymerase. The primers were designed to add 3 stop codons on the extremities of the PCR products.

CysL KO For
TGATTGATTGATCGGCGCGATTCGGCCTCTGG
CysL KO Rev
TCAATCAATCATCGGGTCGCGGTCTCAGGCTC
CysK KO For
TGATTGATTGAAAAACAGTCGGAGGAGTTTCTTGTCC
CysK KO Rev
TCAATCAATCAACTCCCAGTGCCCTCAGCCTC

The PCR products were gel purified using the Nucleospin® Gel and PCR Clean-up kit from Macherey-Nagel and cloned into a pCR2.1-TOPO vector. The construct was

integrated via heat shock into chemically competent *E. coli* HS996 and the selection was done on kanamycin-supplemented LB agar plates. Single colonies were screened for correct constructs via alkaline lysis plasmid preparation and restriction digest by EcoRI. The constructs were then sequenced to ensure the sequence homology.

A correct construct for each KO was transformed into non-methylating chemically competent *E. coli* SCS110. Plasmids were prepared using the GeneJET Plasmid Miniprep kit from Thermo scientific and integrated into MCy8071 via electroporation. Selection of transformed clones was done on kanamycin-supplemented CTT agar plates. KO mutants and wild type cultures were grown in parallel in the presence of an adsorber resin (XAD-16) and samples of crude extracts of the cultures were analysed.

The results showed that in the KO mutants there was a complete absence of cystobactamide production indicating that CysK and CysL are essential for the production of the cystobactamides. Furthermore, the result indicates the essential nature of the cystobactamide biosynthesis gene cluster for the production of the cystobactamides.

Structural analysis:

HRESI(+)MS analysis of cystobactamide A (**1**) returned a pseudomolecular formula ion $(M+H)^+$ consistent with the molecular formula $C_{46}H_{45}N_7O_{14}$, requiring twenty eight double bond equivalents (DBE). The ^{13}C NMR (DMSO-d_6) data revealed seven ester/amide carbonyls (δ_C 163.7 to 169.6) and a further 30 sp^2 resonances (δ_C 114.2 to 150.8), accounting for 22 DBE. Consideration of the 1D and 2D NMR data (Table 1) revealed a set of five aromatic spin systems, three of which were attributed to para-substituted, 1,3,4-trisubstituted and 1,2,3,4-tetrasubstituted benzene rings. A set of HMBC correlations from the aromatic signals H-6,6′ (δ_H 7.96) and the NH (δ_H 8.92) to the amide carbonyl C-4 (δ_C 166.5); NH (δ_H 10.82) to C-7/7′ (δ_C 119.8) and to

the second amide carbonyl C-10 (δ_C 164.6); H-12/12 (δ_H 8.20) to C-10 established the connectivity of two of the *para*-substituted aromatic ring systems (Figure 1). Further examination of the ^{1}H and COSY NMR data established the connectivity of the amide NH (δ_H 8.92) across to the methines H-2 (δ_H 4.96) and H-1 (δ_H 4.70). The downfield characteristic of H-1 (δ_C 79.4) suggested substitution by an oxygen, which was confirmed from a HMBC correlation from H-1 to 1-OMe (δ_H 3.53, δ_C 59.6). Also observed were HMBC correlations from H-1 and H-2 to an ester/amide carbonyl (δ_C 169.6) leading to the construction of subunit A (Figure 1).

For the 1,3,4 trisubstituted benzene ring HMBC correlations were observed from H-17 (δ_H 7.58) to an ester/amide carbonyl C-15 (δ_C 167.3), an oxy quaternary carbon C-18 (δ_C 146.8), C-19 (δ_C 133.6) and C-21 (δ_C 122.9). The isolated spin system for the 1,2,3,4 tetrasubstituted benzene ring showed HMBC correlations from i) H-25 (δ_H 7.82, d, *8.7*) to an ester/amide carbonyl C-23 (δ_C 163.7), C-27 (δ_C 136.2) and a quaternary oxy carbon C-29 (δ_C 150.8); ii) H-26 (δ_H 7.42) to C-24 (δ_C 117.3) and C-28 (δ_C 139.5) along with the phenolic hydroxyl (δ_H 11.25) showing correlations to C-24 and C-28) The tri and tetra-substituted benzene rings were attached *para* to each other by HMBC correlations from the amide NH (δ_H 10.98) to C-20 (δ_C 119.8) C-18 (δ_C 146.7) and C-23 (δ_C 163.7) (Figure 1). The last of the *para*-substituted aromatic spin system H-33/33′ (δ_H 8.11, d, *8.3*) and H-34/34′ (δ_H 7.44, d, *8.3*) showed attachment to the 1,2,3-trisubstituted benzene ring by HMBC correlations of the amide NH (δ_H 9.88) and H-33/33′ to the amide carbonyl C-31 (δ_C 164.3). Additional interpretation of the COSY data revealed two sets of isopropoxy residues (H_3-39 (δ_H 1.38)-H-38 (δ_H 4.76)-H-40 (δ_H 1.38)) and (H_3-42 (δ_H 1.25)-H-41(δ_H 4.30)-H_3-43(δ_H 1.25). The two isopropoxy residues were confirmed to be attached to the oxy quaternary carbons C-18 (δ_C 146.7) and C-28 (δ_C 139.5) based on ROESY correlations from H-38/H-39 to H-17/NH and H-42/43 to NH/29-OH/H-33/33′ (Figure 1). A link between subunit A and B was not established, however based on structural similarity to cystobactamide B, the point of attachment of subunits A and B were inferred. Having accounted for majority of the resonances, $N_2O_3H_2$ and 1DBE were

left to account for. The UV spectrum of the compound showed a λ_{max} of 301 and 320 nm which suggested a conjugated system which was only possible to have been generated by the attachment of a nitro functionality *para*- to the aromatic system on subunit A. The remaining MF was adjusted to generate a carboxylic acid residue (C-15) on the 1,2,3-substituted aromatic ring in subunit B generating the 4-amino-3-isopropoxybenzoic acid moiety leading to the construction of the planar structure of cystobactamide A.

HRESI(+)MS analysis of cystobactamide B (**2**) returned a pseudomolecular formula ion $(M+H)^+$ consistent with the molecular formula $C_{46}H_{44}N_6O_{15}$, requiring twenty eight double bond equivalents (DBE). The NMR data (Table 2) of **2** was highly similar to (**1**) with now the NH (δ_H 10.19) and the oxymethine H-1 (δ_H 4.32) seeing the carbonyl C-37 (δ_C 168.6) confirming the point of attachment of subunits A and B. In addition to this the only change was that the carbonyl amide was now adjusted to a carboxylic acid which was later proven by generation of cystobactamide B dimethyl ester.

HRESI(+)MS analysis of cystobactamide C (**3**) returned a pseudomolecular formula ion $(M+H)^+$ consistent with the molecular formula $C_{27}H_{29}N_3O_7$, requiring 15 (DBE). The ^1H NMR data for cystobactamide C showed aromatic signals which were reminiscent of cystobactamide A and B, however it lacked aromatic resonances for two sets of *para*-substituted aromatic units. The COSY data revealed the existing two sets of isopropoxy residues along with one set of *para*-substituted aromatic ring system. Interpretation of the 1D and 2D NMR data (Table 3, Figure 2) identified cystobactamide C (**3**) bearing resemblance to the eastern part of cystobactamide A and B, consisting of 3-isopropoxybenzoic acid, 2-hydroxy-3-isopropoxybenzamide and a *para*-aminobenzamide unit.

Table 1. NMR (700 MHz, DMSO-d_6) data for cystobactamide A (**1**)

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	HMBC	ROESY
1	4.70, d (*6.9*)	79.4	2	2, 1-OMe, $\underline{C}O_2NH_2$	1-OMe, 3
2	4.96, dd (*8.2*,	55.6	1, 3	1, $\underline{C}O_2NH_2$, 4	1-OMe, 3, 34

	6.9)				
3	8.92, d (*8.2*)		2	4	1, 2, 6′
4		166.5			
5		128.6			
6, 6′	7.96, d (*8.6*)	128.9	7, 7′	4, 6, 6′, 8	3
7, 7′	7.91, d (*8.6*)	119.8	6, 6′	5, 7, 7′	9
8		142.2			
9	10.82, s			7, 7′, 10	7′, 12′
10		164.6			
11		140.4			
12, 12′	8.20, d (*8.6*)	129.5	13, 13′	12, 12′, 10, 14	9
13, 13′	8.39, d (*8.6*)	123.8	12, 12′	11, 13, 13′, 14	
14		149.6			
15		167.3			
16		126.2			
17	7.58, s	114.2		15, 18, 19, 21,	38, 40
18		146.7			
19		133.6			
20	8.50, d (*8.2*)	119.8	21	16, 18	21
21	7.60, d (*8.2*)	122.9	20	15, 17	20
22	10.98, s			18, 20, 23	25, 39
23		163.7			
24		117.3			
25	7.82, d (*8.7*)	125.2	26	23, 24, 29	22
26	7.42[a]	116.3	25	27, 28	30
27		136.2			
28		139.5			
29		150.8			
30	9.88, s			26, 27, 31	33, 41, 42, 43
31		164.3			
32		134.0			
33, 33′	8.11, d (*8.3*)	129.5	34, 34′	31, 33, 33′, 35	30, 41, 42, 43
34, 34′	7.44[a]	125.6	33, 33′	34, 34′, 32	1-OMe, 2
35		137.3			
36	11.53, s				
37		NO			
1-OMe	3.53, s	59.6		1	1, 2
38	4.76, spt (*6.0*)	72.1	39, 40		17
39	1.38, d (*6.0*)	22.1	38	38, 40	22
40	1.38, d (*6.0*)	22.1	38	38, 39	17
41	4.30, spt (*6.0*)	76.0	42, 43		30, 42, 43
42	1.25, d (*6.0*)	22.4	41	41, 43	30, 33′
43	1.25, d (*6.0*)	22.4	41	41. 42	30, 33′
$\underline{C}O_2NH_2$		169.6			
29-OH	11.25, s			27, 28	

[a] Overlapping signals, *Assignments supported by HSQC and HMBC experiments.

Table 2 NMR (700 MHz, DMSO-d_6) data for cystobactamide B (**2**)

pos	δ_H, mult (*J* in Hz)	δ_C	COSY	HMBC	ROESY
1	4.31, m[a]	82.0	2	2, 37, $\underline{C}O_2H$, 1-OMe,	2, 3, 36, 1-OMe
2	5.07, dd (*8.1*, *5.6*)	54.4	1, 3	$\underline{C}O_2H$	1, 1-OMe, 3, 36
3	8.50[b]		2	4	1, 2, 6′
4		166.0			
5		129.3			
6, 6′	7.90, m[c]	128.6	7, 7′	6, 6′, 8	
7, 7′	7.90, m[c]	119.8	6, 6′	7, 7′	9
8		141.7			
9	10.79, s			7, 7′, 10	7′, 12′
10		164.5			
11		140.5			
12, 12′	8.20, d (*8.3*)	129.6	13, 13′	12, 12′, 14, 10	9
13, 13′	8.38, d (*8.3*)	123.8	12, 12′	11, 14, 13, 13′	
14		149.6			
15		167.2			
16		125.9			
17	7.58, s	114.2		15, 18, 19, 21,	38, 40
18		146.6			
19		133.5			
20	8.50[b], d (*8.4*)	119.9	21	16, 18	21
21	7.59, d (*8.4*)	123.0	20	15, 17	
22	10.98, s			20	25, 39
23		163.9			
24		116.8			
25	7.81, d (*8.7*)	125.2	26	23, 29	22
26	7.52, d (*8.7*)	115.6	25	27, 28	30
27		138.8			
28		NO			
29		150.7			
30	9.62, s			31	33, 33′, 26, 41, 43
31		164.5			
32		129.3			
33, 33′	7.97, d (*8.4*)	128.6	34, 34′	31, 33, 33′	30, 41, 42, 43
34, 34′	7.90, m[c]	119.8	33, 33′	34, 34′, 32	1-OMe
35		141.7			
36	10.20, s			34, 37	1, 2, 1-OMe
37		168.6			
1-OMe	3.49, s	59.3		1	1, 2, 34, 36
38	4.75, spt (*6.0*)	72.1	39, 40		17

39	1.38, d (*6.0*)	22.1	38	38, 40	22
40	1.38, d (*6.0*)	22.1	38	38, 39	17
41	4.30, m[a]	76.1	42, 43		30, 42, 43
42	1.25, d (*6.0*)	22.4	41	41, 43	OH
43	1.25, d (*6.0*)	22.4	41	41. 42	OH, 30, 33′
$\underline{C}O_2H$		170.7			
OH	11.22, s			28, 29	

Table 3 NMR (500 MHz, DMSO-d_6) data for cystobactamide C (**3**)

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	HMBC
1		167.3		
2		126.1		
3	7.57, s	114.1		1, 5
4		146.8		
5		133.6		
6	8.49, d (*8.4*)	120.0	7	2, 4
7	7.58, d (*8.4*)	123.0	6	1, 3, 5
8	10.95, s			6
9		164.0		
10		116.0		
11		150.5		
12		137.5		
13		NO		
14	7.65, d (*8.7*)	114.5	15	10, 12
15	7.78, d (*8.7*)	125.3	14	9, 11
16	9.12, s			14, 17
17		164.7		
18		120.4		
19/19′	7.69, d (*8.8*)	129.4	20/20′	19/19′, 21, 17
20/20′	6.62, d (*8.8*)	113.2	19/19′	18, 20/20′
21		152.8		
22	4.75, m	72.0	23/24	
23/24	1.37, d (*6.0*)	22.1	22	23/24
25	4.33, m	75.8	26/27	
26/27	1.28, d (*6.1*)	22.5	25	26/27
OH	11.23, s		25	10

NO-Not Observed, *Assignments supported by HSQC and HMBC experiments

HRESI (+)MS analysis of cystobactamide D (**4**) revealed a pseudomolecular ion ([M + H]$^+$) indicative of a molecular formula ($C_{42}H_{37}O_{14}N_7$) requiring twenty eight double bond equivalents. Interpretation of the NMR (DMSO-d_6) data (Table 4) revealed magnetically equivalent aromatic protons H-12′/12 (δ_H 8.17, d, *8.0*) and H-13/13′ (δ_H 8.36, d, *8.0*) accounting for the first *para*-substitituted benzene ring. Further

interpretation of the ^1H-^1H COSY data revealed the presence of two additional *para*-substituted benzene rings, (H-35/35′) (δ_H 7.80, d, *8.1*) and H-36/36′ (δ_H 7.94, d, *8.1*); the second set of aromatics were heavily overlapped (H-6/6′) and (H-7/7′ (δ_H 7.88). Diagnostic HMBC correlations of the aromatic protons (H-12/12′) to an amide carbonyl C-10 (δ_C 165.1) along with the exchangable (NH) (δ_H 10.82) coupled to C-10, C-7/7′ established the connectivity of the two *para*-substituted aromatic rings (Figure 3), which was further corraborated by ROESY correlations between NH/H-12 and NH/H-7. The COSY data revealed an additional spin system from an oxymethine H-1 (δ_H 4.08, d, *8.0*) through an α-proton H-2 (δ_H 4.91, dd, *8.0*, *7.7*) to an exchangable proton (NH) (δ_H 8.47). HMBC correlations from (i) H-2 to three amide carbonyls C-4 (δ_C 166.4), C-15 (δ_C 171.8) and C-32 (δ_C 169.2), (ii) NH (δ_H 8.48) to C-4, (iii) NH (δ_H 10.54) to C-35/35′ (δ_C 119.5), (iv) H-6/6′ to C-4 further extended the partial structure of cystobactamide D (**4**). Consideration of the 1-D and 2-D NMR data revealed an additional 1,3,4-trisubstituted and a 1,2,3,4-tetrasubstituted benzene ring. HMBC correlations were observed from the aromatic protons H-27 (δ_H 7.55) and H-29 (δ_H 7.60) to the carbonyl C-31 (δ_C 167.8) and the quaternary carbon C-25 (δ_C 133.0), while H-30 (δ_H 8.47, d, *7.0*) and a methoxy signal (δ_H 3.96) were coupled to an oxygen bearing carbon C-26 (δ_C 149.1), hence revealing a 4-amino-3-methoxybenzoic acid moiety, which was later confirmed by esterification. Moreover, HMBC correlations were observed from the exchangeable proton (NH) (δ_H 7.46) to the oxygen bearing carbons C-1 (δ_C 80.8), C-18 (δ_C 141.0) and the aromatic carbon C-22 (δ_C 116.2), while H-22 (δ_H 7.48, d, *8.8*) and the methoxy showed couplings to C-18 and H-21 (δ_H 7.77, d, *8.8*) coupled to an amide carbonyl C-23 (δ_C 164.8). The presence of a hydroxyl functionality *ortho* to the methoxy was later confirmed by esterification (**4a**) (Figure 4), revealing the presence of a 4-amino-2-hydroxy-3-methoxybenzamide. The attachment of the 4-amino-3-methoxybenzoic acid and 4-amin0-2-hydroxy-3-methoxybenzamide substituents were confirmed by ROESY and HMBC correlations from the exchangeable NH's observed from the cystobactamide D dimethyl ester (**4a**). The missing substituents were to be assigned at C-14 (δ_C 150.0) and the carbonyl C-38. The λ_{max} (320 nm) and the downfield chemical shift of

C-14 was suggestive of a nitro substituent at C-14 and the primary amine attached to the carbonyl C-38, generating the planar structure of **4**.

Table 4. NMR (700 MHz, DMSO-d_6) data for cystobactamide D (**4**)

pos	δ_H, mult (*J* in Hz)	δ_C	COSY	ROESY	HMBC
1	4.08, d (*8.0*)	80.7	2		32
2	4.91, dd (*8.0, 7.7*)	56.4	1, 3	33	1, 4, 15, 32
3	8.47[a]		2		4
4		166.4			
5		129.5			
6/6′	7.91, m[b]	129.0	7/7′		4, 8, 6/6′
7/7′	7.91, m[b]	120.4	6/6′		5, 7/7′
8		142.4			
9	10.82, s			12/12′, 7/7′	7, 10
10		165.1			
11		140.9			
12/12′	8.17, d (*8.0*)	129.9	13/13′	9	10, 12/12′, 14
13/13′	8.36, d (*8.0*)	124.3	12/12′	9	11, 13/13′, 14
14		150.0			
15		171.8			
16	NO				
17		129.5			
18		141.0			
19		NO			
20		116.5			
21	7.77, d (*8.8*)	125.8	22		23
22	7.48, d (*8.8*)	115.3	21		18, 20
23		164.8			
24	NO				
25		133.0			
26		149.1			
27	7.55, s	111.7			25, 26, 31
28		126.3			
29	7.60[c], d (*7.0*)	123.3	30		25, 27, 31
30	8.47[a], d, (*7.0*)	120.1	29		26, 28
31		167.8			
32		169.2			
33	10.54, s			2, 35/35′	
34		142.7			
35/35′	7.80, d, (*8.1*)	119.5	36/36′	33	35/35′, 37

36/36′	7.94, d, (*8.1*)	129.3	35/35′	34, 36/36′, 38
37		129.4		
38		165.5		
1-OMe	3.30, s	58.4		1
18-OMe	3.76, s	61.0		18
26-OMe	3.95, s	56.8		26

[a,b,c]overlapping signals, ^{13}C shifts obtained from 2D HSQC and HMBC experiments.

NO-not observed

Table 5. NMR (700 MHz, DMSO-d_6) data for cystobactamide D dimethyl ester (**4a**)

pos	δ_H, mult (*J* in Hz)	δ_C	COSY	ROESY	HMBC
1	4.10[a]	80.4	2	3	2
2	4.92, dd (*8.0, 7.8*)	56.1	1, 3	3, 33	1, 32
3	8.50, d (*7.8*)		2	1, 2, 6/6′	
4		165.6			
5		129.4			
6/6′	7.91, m[b]	128.8	7/7′	3	4, 8
7/7′	7.91, m[b]	120.1	6/6′		
8		142.0			
9	10.82, s			12/12′, 7/7′	7/7′
10		164.8			
11		140.8			
12/12′	8.21, d (*8.7*)	129.7	13/13′	9, 13/13′	10, 12/12′, 14
13/13′	8.39, d (*8.7*)	124.0	12/12′	12/12′	11, 13/13′, 14
14		149.9			
15		NO			
16	9.65, s			18-OMe, 36/36′	38
17		129.5			
18		144.7			
19		152.1			
20		121.8			
21	7.88, d (*8.8*)	126.1	22		19, 23
22	7.95, d (*8.8*)	118.9	21		18, 20
23		162.6			
24	10.94, s			19-OMe	30
25		132.8			
26		148.3			
27	7.60, s	111.2		26-OMe	25, 29, 31
28		124.9			
29	7.67, d (*8.6*)	123.2	30	30	27

30	8.61, d (*8.6*)	119.1	29	29	
31		166.4			
32		169.2			
33	10.59, s			2, 35/35′	
34		142.8			
35/35′	7.83, d, (*8.1*)	119.2	36/36′	33	35/35′, 37
36/36′	7.97, d, (*8.1*)	129.1	35/35′	16	34, 36/36′, 37, 38
37		129.3			
38		165.5			
1-OMe	3.31	58.1			
18-OMe	3.91, s	61.2		16	18
19-OMe	4.10[a], s	62.0		24	19
26-OMe	4.05	56.7		27	
CO_2Me	3.86, s	52.4			31

[a,b]overlapping signals, ^{13}C shifts obtained from 2D HSQC and HMBC experiments. NO-not observed

HRESI (+)MS analysis of cystobactamide E (**5**) revealed a pseudomolecular ion ([M + H]$^+$) indicative of a molecular formula ($C_{26}H_{23}O_9N_5$) requiring eighteen double bond equivalents. The ^1H NMR spectrum was similar to cystobactamide D with the principle difference being the absence of signals reminiscent for the 4-amino-3-methoxybenzoic acid and 4-amino-2-hydroxy-3-methoxybenzamide moieties. Detailed analysis of the 1-D and 2-D NMR data (Table 6) lead to the planar structure of cystobactamide E (**5**).

Table 6. NMR (700 MHz, DMSO-d_6) data for cystobactamide E (**5**)

pos	δ_H, mult (*J* in Hz)	δ_C	COSY	ROESY	HMBC
1	4.08, d (*8.2*)	80.2	2		1-OMe, 2
2	4.90, dd (*8.2, 7.7*)	56.1	1, 3	17	1, 4, 15, 16
3	8.50, d (*7.7*)		2	6/6′	4
4		165.5			
5		129.2			
6/6′	7.91, m[a]	128.6	7/7′	3	4, 6/6′, 8
7/7′	7.91, m[a]	120.0	6/6′	9	5, 7/7′
8		142.0			
9	10.82, s			7/7′, 12/12′	7/7′, 10
10		164.6			

11		140.5			
12/12'	8.21, d (*8.4*)	129.6	13/13'	9	10, 12/12', 14
13/13'	8.38, d (*8.4*)	123.9	12/12'		11, 13/13', 14
14		149.9			
15		171.2			
16		168.9			
17	10.54, s			2, 19/19', 20/20'	16, 19/19'
18		142.8			
19/19'	7.77, d (*8.2*)	119.0	20/20'	17	19/19', 21
20/20'	7.90, m[a]	130.6	19/19'	17	18, 20/20', 22
21		125.6			
22		167.2			
1-OMe	3.29	58.1			1

[a]overlapping signals, ^{13}C shifts obtained from 2D HSQC and HMBC experiments

HRESI(+)MS analysis of cystobactamide F (**6**) returned a pseudomolecular ion $(M+H)^+$ consistent with the molecular Formula $C_{43}H_{39}N_7O_{13}$, requiring 28 DBE. Interpretation of the NMR (DMSO-d_6) data (Table 7) revealed three sets of magnetically equivalent aromatic protons which could be connected via COSY (6/6' and 7/7', 12/12' and 13/13', 33/33' and 34/34') and additionally in contrast to all other cystobactamides a set of magnetically equivalent aromatic protons (26/26' and 27/27') which could be also connected via COSY. These four sets accounted for four *para*-substituted benzene rings in the molecule instead of three as found in all other cystobactamides. Only one 1,2,3,4-tetrasubsituted benzene ring could be detected where HMBC correlations of the aromatic proton H-22 (d_H 7.22) could be observed to the carbon C-18 (d_C 137.1) and C-20 (d_H 114.0) and from the aromatic proton H-21 (d_H 7.51) to C-23 (d_C 167.3). Protons H-21 and H-22 could be connected via COSY correlations. Since carbons C-17, C-19 and C-22 were not observable, the HR-MS/MS mass of all peptide-fragments has been established and revealed the presence of 7 carbons, 11 protons, one nitrogen and three oxygen in the respective fragment, confirming the presence of a 1,2,3,4 substituted *para*-amino benzene moiety on this position (see fig. 1). HMBC data further confirmed the connection of H-37 (d_H 4.93) to C-18 (d_C 137.1). HMBC and COSY data confirmed an identical linker between the two aromatic parts of the molecule as found in cystobactamide D. HMBC correlations from the exchangeable protons H-9 (d_H 10.82) to C-10 (d_C 163.9)

and C-7/7' (d_C 119.4), H-3 (d_H 8.49) to C-4 (d_C 165.1), H-31 (d_H 10.56) to C-30 (d_C 168.3) and C-32 (d_C 141.5) and H-16 (d_H 8.91) to C-36 (d_C 163.1) and C-18 (d_C 137.1) and COSY correlations from H-2 (d_H 4.92) to the exchangeable proton H-3 (d_H 8.49) as well as HRMS fragment data established the serial connectivity of all fragments. The location of the nitro-group and the presence of the free amide group in the linker between the aromatic chains was established using HR-MS/MS fragments to generate the sum-formula of the respective fragments.

Table 7: NMR (700 MHz, DMSO-d_6) data for cystobactamide F (**6**)

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	ROESY	HMBC
1	4.10, d(8.08)	79.7	2	1-OMe, 3	1-OMe, 2, 15, 30
2	4.92, dd(4.10, 4.10)	55.9	1, 3	31	1, 4, 15, 30
3	8.49, d(8.14)		2	1	1, 2, 4
4		165.1			
5		128.7			
6/6'	7.91, m[a]	128.1	7/7'		4, 6/6', 8
7/7'	7.91, m[a]	119.4	6/6'	9	5, 7/7'
8		141.6			
9	10.82, s			7/7', 12/12'	7/7', 8, 10
10		163.9			
11		140			
12/12'	8.21, d(8.71)	129.1	13/13'	9	10, 12/12', 14
13/13'	8.39, d(8.71)	123.3	12/12'		11, 13/13'
14		149			
15		170.6			
16	8.91, s			34/34', 38/38'	18, 36
17		NO			
18		137.1			
19		NO			
20		114.9			
21	7.51, d(9.02)	127.5	22		23
22	7.22, d(9.02)	NO	21		18, 20
23		167.3			
24	15 very broad s				
25		144.5			
26/26'	7.78, d(8.57)	118.4	27/27'		26/26', 28
27/27'	7.86, m[a]	130.1	26/26'		25, 27/27', 29

28		123.4			
29		167.3			
30		168.3			
31	10.56, s			2, 33/33'	30, 33/33'
32		141.5			
33/33'	7.83, m[a]	118.9	34/34'		33/33', 35
34/34'	7.87, m[a]	127.5	33/33'	16	32, 34/34', 36
35		129.2			
36		163.1			
37	4.93, m[a]	71	38/38'		18
38/38'	1.21, d(6.18)	22.4	37	16	37
1-OMe	3.31, s	57.4		1	1

[a] Overlapping signals, NO = Not Observed, * Assignments supported by HSQC and HMBC experiments.

HRESI(+)MS analysis of cystobactamide G (**7**) returned a pseudomolecular ion $(M+H)^+$ consistent with the molecular Formula $C_{44}H_{41}N_7O_{14}$, requiring 28 DBE. Due to overlapping aromatic signals in DMSO-d_6 the NMR data acquired in Methanol-d_4 was used to establish the partial structures of the aromatic and the linker fragment (Table 8). The *para*-substituted benzene rings could be established via COSY, HSQC and HMBC correlations. The configuration of the 1,3,4-trisubstituted benzene ring (4-amino-3methoxy-benzamide) and the methoxy-substituent (1-OMe, (d_C 55.2, d_H 3.50) was established via HSQC, COSY and HMBC correlations. Since not all signals on the 1,2,3,4-substituted benzene moiety could be detected in methanol-d_4 the NMR data measured in DMSO-d_6 was interpreted to establish a 4-amino-3-isopropoxy-2-hydroxy-benzamide and an identical linker between the aromatic parts as identified in cystobactamide D. The connection between C-39 (d_C 74.4) and the carbons C-40/40' (d_C 22.7) was established by COSY correlations of H-39 (d_H 4.82) and H-40/40' (d_H 1.31) and the connectivity between the 1,2,3,4-substituted benzene ring and H-39 (d_H 4.82) was established via HMBC correlations of h-39 to C-18 (d_C 137.3 in DMSO-d_6). The configuration of this benzene moiety was further confirmed with HMBC correlations in DMSO-d_6 of H-22 (d_H 7.04) to C-18 (d_C 137.3) and C-20 (d_C 116.1) and HMBC correlations of H-21 (d_H 7.45) to C-23 (d_C 165.4) as well as COSY correlations from H-21 to H-22. The overall sequence, the location of the nitro-group

and the presence of the free amide group in the linker between the aromatic chains was established using HR-MS/MS fragments to generate the sum-formula of the respective fragments.

Table 8: NMR (700 MHz, Methanol-d_4) data for cystobactamide G (**7**), including (700 MHz, DMSO-d_6) data for pos. 17-23 and 39-40/40'.

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	ROESY	HMBC
1	4.17, d(7.45)	82.1	2		1-OMe, 2, 15, 32
2	5.08, d(7.37)	57.2	1		1, 4, 15, 32
3	NO				
4		168.9			
5		130.5			
6/6'	7.93, m[a]	129.4	7/7'		4, 6/6', 8
7/7'	7.89, d(8.83)	121.1	6/6'		5, 7/7'
8		142.9			
9	NO				
10		166.5			
11		141.6			
12/12'	8.16, d(8.77)	129.9	13/13'		10, 12/12', 14
13/13'	8.38, d(8.74)	124.5	12/12'		11, 13/13'
14		150.9			
15		174.4			
16	NO				
17		139.4			
18	NO	NO			
19		NO			
20		NO			
21	7.74, d(8.83)	125.4	22		23, 17
22	7.51, broad d	NO			
23		168.7			
24	NO				
25		133.5			
26		149.9			
27	7.67, S	112.7			25, 26, 28. 29, 31
28		131.8			
29	7.61, d(8.22)	129.9	30		27, 30, 31
30	8.45, broad d	120.5	29		
31		174.8			
32		169.5			
33	NO				

34		142.8			
35/35'	7.83, d(8.64)	120.8	36/36'		35/35', 37
36/36'	7.93, m[a]	128.9	35/35'		34, 36/36', 38
37		131.2			
38		166.4			
39	4.82, water peak	74.4	40/40'		40
40/40'	1.31, d(6.13)	22.7	39		39
1-Ome	3.50, s	55.2			1
26-Ome	4.02, s	55.9			26

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	ROESY	HMBC
17		NO			
18		137.3			
19		NO			
20		116.1			
21	7.45, d(8.83)	123.9	22		23
22	7.04, d(8.66)	99.7	21		18, 20
23		165.4			
39	5.05, m	69.7	40/40'		18, 40/40'
40/40'	1.17, d(5.98)	22.5	39		39

[a] Overlapping signals, NO = Not Observed, * Assignments supported by HSQC and HMBC experiments.

HRESI(+)MS analysis of cystobactamide H (**8**) returned a pseudomolecular ion $(M+H)^+$ consistent with the molecular Formula $C_{43}H_{39}N_7O_{14}$, requiring 28 DBE. The linker configuration between the aromatic chains was found to be identical as the one found in cystobactamide D. interpretation of HSQC, HMBC and COSY data acquired in DMSO-d_6 revealed three *para*-substituted benzene units as found in cystobactamide A, B, D, F and G. Further interpretation of the COSY, HSGC and HMBC data revealed a identical 1,3,4-trisubstituted benzene moiety which showed HMBC correlations to a methoxy group as found in all other cystobactamides except cystobactamide F (confirmed by HMBC correlation of 1-OMe (d_H 3.27) to C-26 (d_C 147.4)). Analysis of the NMR data revealed – in accordance with the other cystobactamides – a 1,2,3,4-substituted benzene moiety. Significant change came from the establishment of a ethoxy unit via COSY correlation of methylene protons H-39 (d_H 4.17) to methyl group H-40 (d_H 1.27) and the HMBC correlations of methylene

group H-39 (d_H 4.17) to C-18 (d_C 139.5) expanding thereby the substitution pattern of the 4-amino-2-hydroxy-3-X-benzamide moiety to X = methoxy, isoproropoxy or ethoxy on position 3. The sequential sequence of cystobactamide H was established by HMBC correlations of the exchangeable protons H-9 (d_H 10.93) to C-10 (d_C 163.9) and C-7/7' (d_C 119.6), H-33 (d_H 10.85) to C-32 (d_C 168.7) and C-35/35' (d_C 118.8), H-16 (d_H 8.91) to C-38 (d_C 163.1), C-18 (d_C 139.5) and C-22 (d_C 100.4) and H-24 (d_H 14.71) to C-20 (d_C 116.1), C-25 (d_C 131.0), C-26 (d_C 147.4) and C-30 (d_C 118.5) and H-2 (d_H 4.85) to C-4 (d_C 165.5) as well as HR-MS2 fragmentation-data which also enabled the localisation of the nitro-group and the establishment of the free amide group in the linker moiety.

Table 9: NMR (700 MHz, DMSO-d_6) data for cystobactamide H (**8**)

pos	δ_H, mult (*J* in Hz)	δ_C*	COSY	ROESY	HMBC
1	4.22, d (8.60)	79.8	2	3, 33	2, 32, 1-OMe
2	4.85, dd (8.42, 8.42)	56.3	1, 3	3, 33	1, 4, 15, 32
3	9.02 s		2		
4		165.5			
5		128.8			
6/6'	7.93 m[a]	128.3	7/7'		4, 6/6', 8
7/7'	7.91 m[a]	119.6	6/6'		5, 7/7'
8		141.7			
9	10.93 s			7/7', 12/12'	
10		163.9			
11		140.3			
12/12'	8.22, d(8.72)	129.4	13/13'		10, 12/12', 14
13/13'	8.38, d(8.72)	123.5	12/12'		11, 13/13'
14		149.2			
15		170.7			
16	8.91 s			22, 39, 40	18, 22, 38
17		NO			
18		139.5			
19		NO			
20		116.1			
21	7.45, d(8.63)	124.1	22		18, 23
22	6.95, d(8.66)	100.4	21	16	18
23		165.8			
24	14.71 s			26-OMe, 39	23, 25, 26, 30

25		131.0			
26		147.4			
27	7.46, s	111.1			25, 26, 29, 28, 31
28		133.9			
29	7.38, m[a]	121.3	30		27, 28, 30
30	8.44, d(8.29)	118.5	29		25, 26, 28,
31		169.9			
32		168.7			
33	10.85 s			1, 2, 35/35'	35/35'
34		141.9			
35/35'	7.85, m[a]	118.8	36/36'		37
36/36'	7.85, m[a]	127.7	35/35'		34, 38
37		129.5			
38		163.1			
39	4.17, q(7.03)	65.4	40		18, 40
40	1.27, t(7.07)	15.7	39		39
1-Ome	3.27, s	57.4			1
26-Ome	3.84, s	55.2			26

[a] Overlapping signals, NO = Not Observed, * Assignments supported by HSQC and HMBC experiments.

Figures:

Figure 1: Key 2D NMR correlations (700 MHz, DMSO-d_6) for cystobactamide A (**1**)

Figure 2: Key 2D NMR correlations (500 MHz, DMSO-d_6) for cystobactamide C (**3**)

Figure 3: Key 2D NMR correlations (700 MHz, DMSO-d_6) for cystobactamide D (**4**)

Figure 4: Key 2D NMR correlations of cystobactamide D dimethyl ester (**4a**)

Figure 5: Key 2D NMR correlations of cystobactamide E (**5**)

Figure 6: Key 2D NMR correlations (700 MHz, DMSO-d_6) of cystobactamide F (**6**)

Figure 7: Key 2D NMR correlations (700 MHz, MeOH-d_4) of cystobactamide G (**7**)

Figure 8: Key 2D NMR correlations (700 MHz, DMSO-d_6) of cystobactamide H (**8**)

Biological evaluation of cystobactamides

As summarized in Tables 10a/b, cystobactamides were evaluated against several microorganisms and cell lines. All derivatives demonstrated a potent inhibitory effect on various *E. coli* strains, including a nalidixic acid resistant (NAL^R) isolate. Overall potency (average MIC values) of the tested derivatives increased in the following

order: CysA1, CysC < CysB < CysA, CysG < CysF. Importantly, the pathogenic Gram-negative strains *A. baumannii* and *P. aeruginosa* were also inhibited by the most active derivatives, CysA, CysB, CysG, and CysF, in the low µg/ml range, which is in terms of MIC values only by one order of magnitude higher than for the reference drug ciprofloxacin.

Average MIC values on Gram-positive bacteria, such as *E. faecalis*, *S. aureus*, and *S. pneumonia* were partly in the sub-µg/ml range and the average potency of CysA and CysB exceeded that of ciprofloxacin.

Furthermore, it was shown that cystobactamides do not inhibit the growth of yeast and mammalian cells, respectively. Thus, the cystobactamides did not cause apparent cytotoxicity.

Susceptibility of mutant *E. coli* strains to cystobactamides

Quinolones are a widely used class of antibiotics that target the type II topoisomerases, DNA gyrase and topoisomerase IV. Resistance to quinolones is thereby often mediated by mutations in chromosomal genes that lead to alterations in the drug targets. In GyrA the quinolone-resistance determining region (QRDR) is located between amino acids 67 and 106, whereas amino acids 83 (Ser) and 87 (Asp) are most often involved.[1,2] In analogous regions of ParC, the secondary target of quinolones, changes of amino acid 80 (Ser) are found to confer quinolone resistance.[3,4]

Cystobactamides were screened using a panel of *E. coli* strains with typical mutations in *gyrA* and *parC* genes (Table 11). With ciprofloxacin the MIC values increase approximately by factor 30 for the single-step *gyrA* mutations (strain MI and WT-3.2). However, a combination of both *gyrA* mutations (strain WT-3) results already in nearly clinical resistance (1 mg/L). A *parC* mutation (strain WT-4 M2.1) leads to a two-fold increase of the MIC of ciprofloxacin. However, MIC values for cystobactamides did not or only marginally increase for *gyrA* and *parC* mutant *E. coli* strains, which suggests that cystobactamides might interfere with amino acids 87 and

83 of GyrA and amino acid 80 of ParC to a lower extent than observed for ciprofloxacin.

High-level quinolone resistance often results from a combination of several target site mutations and altered efflux mechanisms. The *in vitro* selected mutant WT III (*marR* Δ74bp) does not produce functional MarR, which acts as a repressor of *marA* expression. This, in turn, leads to overproduction of MarA and AcrAB and overexpression of the AcrAB efflux pump is associated with the MAR (multiple antibiotic resistance) phenotype.[5] *E. coli* strain WT III was less susceptible to ciprofloxacin treatment by a factor of ca. 4 (cp. *E. coli* WT). In comparison, MIC values of cystobactamides B, F, and G were still in the µg/ml range. Notably, the MIC of CysF on strain *E. coli* WT III only increased by factor 2 compared to wildtype *E. coli* DSM-1116, whereas the MIC of ciprofloxacin increased by ca. factor 10.

Table 10a: Antimicrobial activity of cystobactamides (Cys).

Test organism	CysA	CysA1	CysB	CysC
	MIC [µg/ml]			
Acinetobacter baumannii DSM-30008	7,4	58,9	3,7	32,5
Burkholderia cenocepacia DSM-16553	> 59	> 59	> 59	> 65
Chromobacterium violaceum DSM-30191	> 59	> 59	14,7	16,3
Escherichia coli DSM-1116	0,9	14,7	1,8	16,3
Escherichia coli DSM-12242 (NAL^R)	0,9	29,4	3,7	8,1
Escherichia coli DSM-26863 (*tolC3*)	0,5	7,4	1,8	4,1
Escherichia coli ATCC35218	0,9	14,7	1,8	16,3
Escherichia coli ATCC25922	0,5	7,4	0,9	8,1
Enterobacter aerogenes DSM-30053	> 59	> 59	> 59	> 33
Klebsiella pneumoniae DSM-30104	> 59	> 59	> 59	65
Pseudomonas aeruginosa PA14	> 59	58,9	14,7	65
Pseudomonas aeruginosa ATCC27853	> 59	58,9	14,7	65
Mycobacterium smegmatis mc^2155 ATCC700084	> 59	> 59	> 59	> 65
Bacillus subtilis DSM-10	0,12	1,8	0,46	2,0

Enterococcus faecalis ATCC29212	0,06	3,7	0,23	4,1
Micrococcus luteus DSM-1790	0,06	7,4	0,23	4,1
Staphylococcus aureus ATCC29213	0,12	14,7	0,12	8,1
Streptococcus pneumoniae DSM-20566	0,23	14,7	0,46	8,1
Candida albicans DSM-1665	> 59	> 59	> 59	> 65
Pichia anomala DSM-6766	> 59	> 59	> 59	> 65

Test organism	CysF	CysG	CIP
Acinetobacter baumannii DSM-30008	-	-	0,2
Burkholderia cenocepacia DSM-16553	-	-	6,4
Chromobacterium violaceum DSM-30191	-	-	0,006
Escherichia coli DSM-1116	0,4	0,9	0,006
Escherichia coli DSM-12242 (NAL^R)	-		0,05
Escherichia coli DSM-26863 (*tolC3*)	0,4	0,9	≤ 0,003
Escherichia coli ATCC35218	-	-	0,006
Escherichia coli ATCC25922	-	-	≤ 0,003
Enterobacter aerogenes DSM-30053	-	-	0,2
Klebsiella pneumoniae DSM-30104	-	-	0,025
Pseudomonas aeruginosa PA14	3,4	7,1	0,1
Pseudomonas aeruginosa ATCC27853	-	-	0,1
Mycobacterium smegmatis mc^2155 ATCC700084	-	-	0,4
Bacillus subtilis DSM-10	-	-	0,1
Enterococcus faecalis ATCC29212	-	-	0,8
Micrococcus luteus DSM-1790	-	-	1,6
Staphylococcus aureus ATCC29213	-	-	0,1
Streptococcus pneumoniae DSM-20566	-	-	1,6
Candida albicans DSM-1665	-	-	> 6,4
Pichia anomala DSM-6766	-	-	> 6,4

CIP reference antibiotic ciprofloxacin

- not determined

Table 10b: Cytotoxicity of cystobactamides (Cys).

Cell lines and primary cells	GI_{50} [µM]		
	CysA	CysA1	CysB
CHO-K1 (Chinese hamster ovary)	37 -111	> 111	> 111
HCT-116 (human colon carcinoma)	-	-	> 50
HUVEC (human umbilical vein endothelial cells)	-	-	> 50

Cell lines and primary cells	GI_{50} [µM]		
	CysC	CysF	CysG
CHO-K1 (Chinese hamster ovary)	ca. 111	> 111	37 -111
HCT-116 (human colon carcinoma)	-	-	-
HUVEC (human umbilical vein endothelial cells)	-	-	-

- not determined

Table 11: Antimicrobial activity of cystobactamides (Cys) against *E. coli* mutant strains.

Test organism [resistance mutations]	**CysA**	**CysA1**	**CysB**	**CysC**
	MIC [µg/ml]			
Escherichia coli WT	0,5	14,7	1,8	8,1
Escherichia coli MI [*gyrA*(S83L)]	3,7	29,4	3,7	16,3
Escherichia coli WT-3.2 [*gyrA*(D87G)]	3,7	29,4	3,7	32,5
Escherichia coli WT-3 [*gyrA*(S83L, D87G)]	14,7	> 59	7,4	> 33
Escherichia coli WT-4 M2.1 [*parC*(S80I)]	0,5	14,7	1,8	8,1
Escherichia coli MI-4 [*gyrA*(S83L), *parC*(S80I)]	0,5	14,7	1,8	16,3
Escherichia coli WTIII [marRΔ74bp]	14,7	58,9	3,7	65

Test organism [resistance mutations]	**CysF**	**CysG**	**CIP**
	MIC [µg/ml]		
Escherichia coli WT	-	-	0,013
Escherichia coli MI [*gyrA*(S83L)]	-	-	0,4
Escherichia coli WT-3.2 [*gyrA*(D87G)]	-	-	0,4
Escherichia coli WT-3 [*gyrA*(S83L, D87G)]	-	-	0,8
Escherichia coli WT-4 M2.1 [*parC*(S80I)]	-	-	0,025
Escherichia coli MI-4 [*gyrA*(S83L), *parC*(S80I)]	-	-	0,4
Escherichia coli WTIII [marRΔ74bp]	0,9	3,6	0,05

CIP reference antibiotic ciprofloxacin
- not determined

Experimental Procedures Cell-Based Assays

Cell lines and primary cells. Human HCT-116 colon carcinoma cells (CCL-247) were obtained from the American Type Culture Collection (ATCC) and Chinese

hamster ovary CHO-K1 cells (ACC-110) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Both cell lines were cultured under the conditions recommended by the respective depositor. Primary HUVEC (human umbilical vein endothelial cells; single donor) were purchased from PromoCell (Heidelberg, Germany) and cultured in Endothelial Cell Growth Medium (PromoCell) containing the following supplements: 2% FCS, 0.4% ECGS, 0.1 ng/ml EGF, 1 ng/ml bFGF, 90 µg/ml heparin, 1 µg/ml hydrocortisone.

Bacterial strains. Bacterial wildtype strains used in susceptibility assays were either part of our strain collection or purchased from the German Collection of Microorgansims and Cell Cultures (DSMZ) or from the American Type Culture Collection (ATCC). *E. coli* strain WT[6] and *E. coli* mutants were kindly provided by Prof. Dr. P. Heisig, Pharmaceutical Biology and Microbiology, University of Hamburg.

Cytotoxicity assay. Cells were seeded at 6 x 10^3 cells per well of 96-well plates (Corning CellBind®) in complete medium (180 µl) and directly treated with cystobactamides dissolved in methanol in a serial dilution. Compound were tested in duplicate for 5 d, as well as the internal solvent control. After 5 d incubation, 5 mg/ml MTT in PBS (20 µL) was added per well and it was further incubated for 2 h at 37 °C.[7] The medium was then discarded and cells were washed with PBS (100 µl) before adding 2-propanol/10N HCl (250:1, v/v; 100 µl) in order to dissolve formazan granules. The absorbance at 570 nm was measured using a microplate reader (EL808, Bio-Tek Instruments Inc.).

Susceptibility testing. MIC values were determined in microdilution assays. Overnight cultures were diluted in the appropriate growth medium to achieve an inoculum of 10^4-10^6 cfu/mL. Yeasts were grown in Myc medium (1% phytone peptone, 1% glucose, 50 mM HEPES, pH 7.0), *S. pneumonia* and *E. faecalis* in tryptic soy broth (TSB: 1.7% peptone casein, 0.3% peptone soymeal, 0.25% glucose, 0.5% NaCl, 0.25% K_2HPO_4; pH 7.3); *M. smegmatis* in Middlebrook 7H9 medium supplemented with 10% Middlebrook ADC enrichment and 2 ml/l glycerol). All other listed bacteria were grown in Müller-Hinton broth (0.2% beef infusion solids, 1.75%

casein hydrolysate, 0.15% starch, pH 7.4). Cystobactamides and reference drugs were added directly to the cultures in sterile 96-well plates as duplicates and serial dilutions were prepared. Microorganisms were grown on a microplate shaker (750 rpm, 30-37 °C, 18-48 h), except *S. pneumonia*, which was grown at non-shaking conditions (37 °C, 5% CO_2, 18 h). Growth inhibition was assessed by visual inspection and the MIC was defined as the lowest concentration of compound that inhibited visible growth.

Target identification

To test the anti-gyrase activity of cystobactamides, commercial *E. coli* gyrase supercoiling kits (Inspiralis) were used. Cystobactamide A inhibited the E. coli gyrase (20,5 nM eq. 1 unit) showing an apparent IC_{50} of 6 µM. Cystobactamide A1 inhibited the E. coli gyrase (20,5 nM eq. 1 unit) showing an apparent IC_{50} of 2.5 µM. Cystobactamide D inhibited the E. coli gyrase (20,5 nM eq. 1 unit) showing an apparent IC_{50} of 1 µM. Cystobactamide C inhibited the *E. coli* gyrase (20,5 nM eq. 1 unit) showing an apparent IC_{50} of 7,7 µM. Cystobactamides thus are novel inhibitors of bacterial DNA gyrase.

IC_{50} values of cystobactamide A-D in the Gyrase inhibition assay:

Compound	IC_{50}/µM
cystobactamide A	6 +/- 1.4
cystobactamide A1	2.5 +/- 0,8
cystobactamide C	7.2 +/- 0.74
cystobactamide D	0.7 +/- 0.4

Figure 9a show the results of the Gyrase inhibition assay. The gyrase reactions were titrated with varying concentrations of cystobactamide A, A1, C and D and resolved by agarose gel electrophoresis. For IC_{50} determination the band intensity of the

supercoiled plasmid was determined using Adobe Photoshop, plotted vs. [cystobactamide] and fitted using Hill's equation.

Prokaryotic DNA gyrase and topoisomerase IV share a high degree of homology and gyrase inhibitors typically show a topoisomerase IV inhibitory activity.[8] To test the influence of the cystobactamides on topoisomerase IV a commercial *E. coli* topoisomerase IV kit (Inspiralis) was used.

Cystobactamide A inhibited the activity of *E. coli* topo IV only at the highest tested concentration of 815 µM. Cystobactamide A1 inhibited *E. coli* topo IV showing an IC_{50} value of 6.4 +/- 1.8 µM. Cystobactamide C inhibited the activity of *E. coli* topo IV only at the highest tested concentration of 300 µM. Cystobactamide D inhibited *E. coli* topo IV showing an IC_{50} value of 10 +/- 3 µM.

IC_{50} values for cystobactamide A-D in the *E. coli* Topoisomerase IV inhibition assay:

Compound	IC_{50}/µM
cystobactamide A	>160
cystobactamide A1	6.4 +/- 1.8
cystobactamide C	>60
cystobactamide D	10 +/- 3

Figure 9b shows the result of the Topoisomerase IV inhibition assay. The topo IV reactions were titrated with varying concentrations of A-D and resolved by agarose gel electrophoresis. For IC_{50} determination the band intensity of the supercoiled plasmid was determined using Adobe Photoshop, plotted vs. [cystobactamide] and fitted using Hill's equation.

Prokaryotic DNA topoisomerase IV and eukaryotic topoisomerase II share a high degree of homology (type IIa topoisomerases) and inhibitors of the prokaryotic enzyme often also inhibits the eukaryotic counterpart.[8] To test the influence of the

cystobactamides on eukaryotic topoisomerase IV a commercial *H. sapiens* topoisomerase II kit (Inspiralis) was used.

Cystobactamide A inhibited the activity of human topo II only at the highest tested concentration of 815 µM. Cystobactamide A1 inhibited human topo II showing an IC_{50} value of 9 +/- 0.03 µM. Cystobactamide C inhibited the activity of human topo II only at the highest tested concentration of 300 µM. Cystobactamide D inhibited human topo II showing an IC_{50} value of 41.2 +/- 3 µM

IC_{50} values for cystobactamide A-D in the *H. sapiens* Topoisomerase II inhibition assay:

Compound	IC_{50}/µM
cystobactamide A	>160
cystobactamide A1	9 +/- 0.03
cystobactamide C	>60
cystobactamide D	41.2 +/- 3

Figure 9c shows the result of the Topoisomerase II inhibition assay. The topo II reactions were titrated with varying concentrations of A-D and resolved by agarose gel electrophoresis. For IC_{50} determination the band intensity of the supercoiled plasmid was determined using Adobe Photoshop, plotted vs. [cystobactamide] and fitted using Hill's equation.

Aside the ATP-dependent type IIa topoisomerases like E. coli gyrase, topoIV and human topoII, the activity of cystobactamides on the ATP-independent human topoisomerase I was tested as well.

IC_{50} values for cystobactamide A-D in the *H. sapiens* Topoisomerase I inhibition assay:

Compound	IC_{50}/µM

cystobactamide A	~10
cystobactamide A1	~0.7
cystobactamide C	~6
cystobactamide D	~33.6

Figure 9d shows the result of the Topoisomerase I inhibition assay. The topo I reactions were titrated with varying concentrations of A-D and resolved by agarose gel electrophoresis. For IC_{50} determination the band intensity of the supercoiled plasmid was determined using Adobe Photoshop, plotted vs. [cystobactamide] and fitted using Hill's equation.

IC_{50}(gyrase) vs. IC_{50}(topoisomerase IV) value comparison of cystobactamide A-D:

	IC_{50}/µM		ratios
ratios	gyrase	Topo IV	IC_{50}(topo IV)/ IC_{50}(gyrase)
cystobactamide A	6	~815	~136
cystobactamide A1	2.5	6.4	~2.6
cystobactamide D	0.7	10	~14
cystobactamide C	7.2	~300	~42

Cystobactamides A and C show a strong preference for gyrase as molecular target (40-100 fold stronger preference for gyrase). A1 and D both target gyrase and topoisomerase IV almost equally well (2.6-10 fold stronger preference for gyrase).

Generally, there are two described inhibition modes/binding sites for gyrase inhibitors:

1. Compounds like the fluoroquinolones bind to the GyrA DNA complex and avoid the religation of the nicked dsDNA (gyrase poisoning); and
2. Aminocoumarins on the other hand bind to the ATP binding pocket on GyrB (competitive inhibition).[8]

To test if cystobactamides follow any of those two inhibition modes, DNA/gyrase complex linearization assays (A) and ATP competition assays (B) were performed using cystobactamide D. (A) Here, the complex of DNA and gyrase is trapped using SDS and the gyrase is digested using proteinase K. If the gyrase/DNA complex is trapped by a gyrase inhibitor of type 1 this will lead to the formation of linearized plasmid (as the religation is inhibited). Type 2 inhibitor-bound or compound-free samples will not show the formation of linearized plasmids. The results of the assay are shown in Figure 10a. Ciprofloxacin (a known gyrase/DNA stabilizer) and cystobactamide D show the formation of linearized plasmid after proteinase K treatment. This effect is not seen for the untreated control. Therefore, it appears likely that cystobactamides stabilize the covalent GyrA-DNA complex in a fashion comparable to the fluoroquionolones. (B) Here, standard gyrase reactions were inhibited using a constant amount of cystobactamide D and titrated with increasing amounts of ATP. If ATP and cystobactamide D would compete for binding at the ATP binding pocket on the gyrase GyrB subunit, increasing amounts of ATP would lead to the formation of supercoiled plasmid in the assay. Figure 10b shows the assay results. Even at the highest ATP concentration of 10 mM (2000 fold cystobactamide concentration) the gyrase activity is not regained, indicating that the ATP binding pocket is not the binding site of the cystobactamides. This result is in line with the linearization assay results.

Figure 11 shows the results of the DNA/gyrase complex linearization assay.

Experimental procedures

Gyrase supercoiling assay

To test the anti-gyrase activity of cystobactamides, commercial E. coli gyrase supercoiling kits (Inspiralis, Norwich, UK) were used.3 For standard reactions 0.5 µg relaxed plasmid were mixed with 1 unit (~20.5 nM) E. coli gyrase in 1x reaction buffer (30 µl final volume, see kit manual) and incubated for 30 minutes at 37°C. The reactions were quenched by the addition of DNA gel loading buffer containing 10%

(w/v) SDS. The samples were separated on 0.8% (w/v) agarose gels and DNA was visualized using Roti-GelStain (Carl Roth).

All natural products stock solutions and dilutions were prepared in 100% DMSO and added to the supercoiling reactions giving a final DMSO concentration of 5% (v/v). Ciprofloxacin stock solutions and Dilutions were prepared in 10 mM HCl and 50 % DMSO and used 1:10 in the final assay.

Following natural product concentrations were used in the assay:
Cystobactamide A: 815.8 µM; 163 µM; 80 µM, 16 µM; 8 µM; 1.6 µM; 0.8 µM; 0.16 µM; 0.08 µM; 0.016 µM
Cystobactamide A1: 543.5 µM; 108.7 µM; 54 µM; 10.8 µM; 5.4 µM; 1.087 µM; 0.54 µM; 0.108 µM; 0.054 µM; 0.0108 µM
Cystobactamide C: 300 µM; 60 µM; 30 µM; 6 µM; 3 µM; 0.6 µM; 0.3 µM; 0.06 µM; 0.03 µM; 0.006 µM
Cystobactamide D: 347 µM; 173.5 µM; 86.75 µM; 43.38 µM; 21.69 µM; 10.84 µM; 5.42 µM; 2.71 µM; 1.36 µM; 0.68 µM; 0.34 µM; 0.17 µM; 0.085 µM; 0.042 µM; 0.021µM; 0.0106 µM; 0.0053 µM

Control reactions were: no enzyme and a standard reaction in presence of 5% (v/v) DMSO.

All reaction samples were equilibrated for 10 minutes at room-temperature in the absence of DNA. Then the relaxed plasmid was added to start the reaction.

Proteinase K linearization assay

To test if cystobactamides stabilize the covalent complex between DNA gyrase and the nicked DNA substrate, proteinase K linearization assay were performed (see a). Standard gyrase supercoiling assays were run in the presence of cystobactamide D (18 µM; 1.8 µM). Control reactions contained no gyrase, no inhibitor or the known gyrase/DNA complex stabilizer ciprofloxacin (1 µM). The reactions were quenched by the addition of 1/10 volume of 10% SDS. To linearize the nicked DNA-gyrase

complexes, 50 µg/ml proteinase K were added to the reactions and incubated for 30 minutes at 37°C. The samples were separated on 0.8% (w/v) agarose gels and DNA was visualized using Roti-GelStain (Carl Roth). To detect linearized plasmid bands the relaxed plasmid was digested by the single-cutting restriction enzyme NdeI.

Gyrase supercoiling assay with varying ATP concentrations

To test if cystobactamides compete with ATP for binding to the ATP binding pocket on GyrB, standard gyrase supercoiling assays (see a) with varying ATP concentrations were performed. Standard reaction mixes (1 mM ATP) were supplemented with ATP (0.5M ATP stock solution, ATP was purchased from Sigma-Aldrich) to final ATP concentrations of 2.5; 5 and 10 mM. All reactions were performed in triplicates.

Topoisomerase IV relaxation assay

To test the anti-topoisomerase IV activity of cystobactamides, commercial E. coli topoisomerase IV relaxing kits (Inspiralis, Norwich, UK) were used.4 For standard reactions 0,5 µg supercoiled plasmid were mixed with 1 unit (~20.5 nM) E. coli topoisomerase IV in 1x reaction buffer (see kit manual) and incubated for 30 minutes at 37°C. The reactions were quenched by the addition of DNA gel loading buffer containing 10% (w/v) SDS. The samples were separated on 0.8% (w/v) agarose gels and DNA was visualized using Roti-GelStain (Carl Roth).

Following natural product concentrations were used in the assay:

Cystobactamide A: 815.8 µM; 163 µM; 80 µM, 16 µM; 8 µM; 1.6 µM; 0.8 µM; 0.16 µM; 0.08 µM; 0.016 µM

Cystobactamide A1: 543.5 µM; 108.7 µM; 54 µM; 10.8 µM; 5.4 µM; 1.087 µM; 0.54 µM; 0.108 µM; 0.054 µM; 0.0108 µM

Cystobactamide C: 300 µM; 60 µM; 30 µM; 6 µM; 3 µM; 0.6 µM; 0.3 µM; 0.06 µM; 0.03 µM; 0.006 µM

Cystobactamide D: 347 µM; 173.5 µM; 86.75 µM; 43.38 µM; 21.69 µM; 10.84 µM; 5.42 µM; 2.71 µM; 1.36 µM; 0.68 µM; 0.34 µM; 0.17 µM; 0.085 µM; 0.042 µM; 0.021µM; 0.0106 µM; 0.0053 µM

Control reactions were: no enzyme and a standard reaction in presence of 5% (v/v) DMSO. All reaction samples were equilibrated for 10 minutes at room-temperature in the absence of DNA. Then the relaxed plasmid was added to start the reaction.

Topoisomerase II relaxation assay

To test the anti-topoisomerase II activity of cystobactamides, commercial human topoisomerase IV relaxing kits (Inspiralis, Norwich, UK) were used.4 For standard reactions 0,5 µg supercoiled plasmid were mixed with 1 unit (~20.5 nM) E. coli topoisomerase II in 1x reaction buffer (see kit manual) and incubated for 30 minutes at 37°C. The reactions were quenched by the addition of DNA gel loading buffer containing 10% (w/v) SDS. The samples were separated on 0.8% (w/v) agarose gels and DNA was visualized using Roti-GelStain (Carl Roth).

Following natural product concentrations were used in the assay:
Cystobactamide A: 815.8 µM; 163 µM; 80 µM, 16 µM; 8 µM; 1.6 µM; 0.8 µM; 0.16 µM; 0.08 µM; 0.016 µM
Cystobactamide A1: 543.5 µM; 108.7 µM; 54 µM; 10.8 µM; 5.4 µM; 1.087 µM; 0.54 µM; 0.108 µM; 0.054 µM; 0.0108 µM
Cystobactamide C: 300 µM; 60 µM; 30 µM; 6 µM; 3 µM; 0.6 µM; 0.3 µM; 0.06 µM; 0.03 µM; 0.006 µM
Cystobactamide D: 347 µM; 173.5 µM; 86.75 µM; 43.38 µM; 21.69 µM; 10.84 µM; 5.42 µM; 2.71 µM; 1.36 µM; 0.68 µM; 0.34 µM; 0.17 µM; 0.085 µM; 0.042 µM; 0.021µM; 0.0106 µM; 0.0053 µM

Control reactions were: no enzyme and a standard reaction in presence of 5% (v/v) DMSO. All reaction samples were equilibrated for 10 minutes at room-temperature in the absence of DNA. Then the relaxed plasmid was added to start the reaction.

Topoisomerase I relaxation assay

To test the anti-topoisomerase II activity of cystobactamides, commercial *H. sapiens* topoisomerase I relaxing kits (Inspiralis, Norwich, UK) were used.4 For standard

reactions 0,5 µg supercoiled plasmid were mixed with 1 unit (~20.5 nM) H. sapiens topoisomerase I in 1x reaction buffer (see kit manual) and incubated for 30 minutes at 37°C. The reactions were quenched by the addition of DNA gel loading buffer containing 10% (w/v) SDS. The samples were separated on 0.8% (w/v) agarose gels and DNA was visualized using Roti-GelStain (Carl Roth).

Following natural product concentrations were used in the assay:
Cystobactamide A: 815 µM; 81.5 µM; 8.15 µM
Cystobactamide A1: 543 µM; 54.3 µM; 5.43 µM
Cystobactamide C: 300 µM; 30 µM; 3 µM
Cystobactamide D: 277 µM; 27.2 µM; 2.77 µM

Control reactions were: no enzyme and a standard reaction in presence of 5% (v/v) DMSO. All reaction samples were equilibrated for 10 minutes at room-temperature in the absence of DNA. Then the relaxed plasmid was added to start the reaction

Quantification and analysis

To determine IC50 values, the formation of supercoiled (gyrase) or relaxed (topoisomerase I, II IV) plasmid was quantified using Adobe Photoshop (Histogram mode). Plotting of these values versus the compound concentration yielded sigmoidal shaped curves, which were fitted using Hill's equation (Origin Pro 8.5). All determined IC50 values are the averages of three independent experiments.

References:

[1] T. Gruger, J. L. Nitiss, A. Maxwell, E. L. Zechiedrich, P. Heisig, S. Seeber, Y. Pommier, D. Strumberg, *Antimicrob. Agents Chemother. 48*, **2004**, 4495-4504.

[2] H. Schedletzky, B. Wiedemann, P. Heisig, *J. Antimicrob. Chemother. 43*, **1999**, 31-37.

[3] A. B. Khodursky, E. L. Zechiedrich, N. R. Cozzarelli, *Proc. Natl. Acad. Sci. USA 92*, **1995**, 11801-11805.

[4] A. Schulte, P. Heisig, *J. Antimicrob. Chemother. 46*, **2000**, 1037-1046.

[5] D. Keeney, A. Ruzin, F. McAleese, E. Murphy, P. A. Bradford, *J. Antimicrob. Chemother. 61*, **2008**, 46-53.

[6] P. Heisig, H. Schedletzky, H. Falkenstein-Paul, *Antimicrob. Agents Chemother. 37*, **1993**, 669-701.

[7] T. Mosmann, *J. Immunol. Meth. 65*, **1983**, 55-63.

[8] Pommier, Y.; Leo, E.; Zhang, H.; Marchand, C. *Chemistry & Biology* **2010**, *17*, 421.

Synthesis of cystobactamide A and C

First, the synthesis of cystobactamide C is described which can further be elaborated to the other cystobactamides.

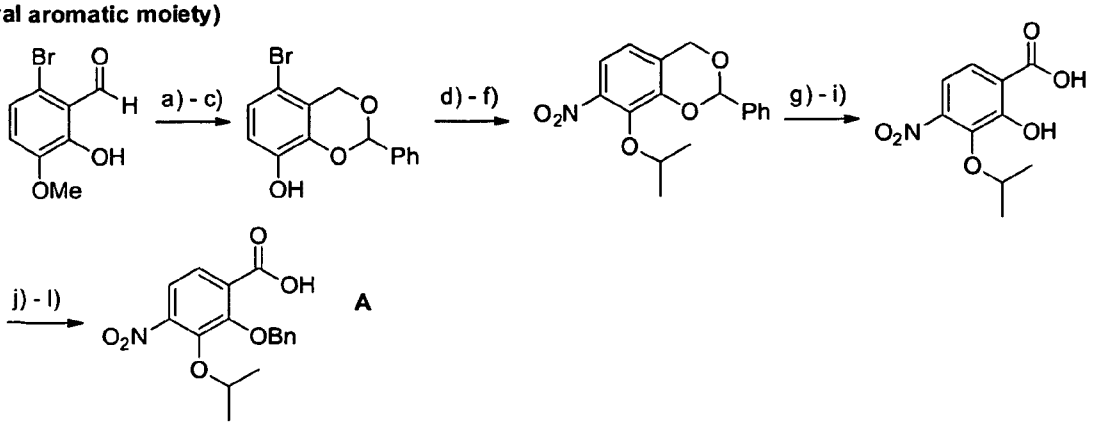
1.1. Cystobactamide C

The following Schemes 1 and 2 provide an overview on the synthesis of individual aromatic building blocks followed by assembling these to generate cystobactamide C.

Alternatively, step e) in Scheme 1 can be modified by using another alcohol (R'OH) instead of ^{i}PrOH. If for example EtOH is used, building blocks of cystobactamide H can be prepared. The same applies for step b) in the second reaction sequence given in Scheme 1. Here, also ^{i}PrOH can be exchanged by any other alcohol (R'OH). If for example MeOH is used, building blocks of cystobactamides C, G and H can be prepared. For the preparation of cystobactamide F, p-amino-benzoic acid derivatives such as p-aminobenzoic acid or corresponding N-protected aminobenzoic acid derivatives and p-nitrobenzoic acids are employed instead of building block B.

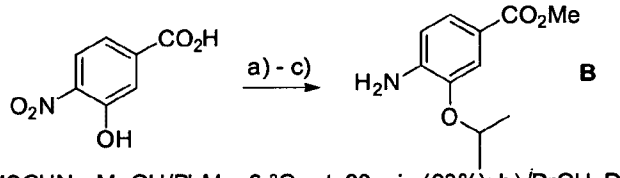
Scheme 1: Syntheses of arenes A and B followed by amide coupling.

(central aromatic moiety)



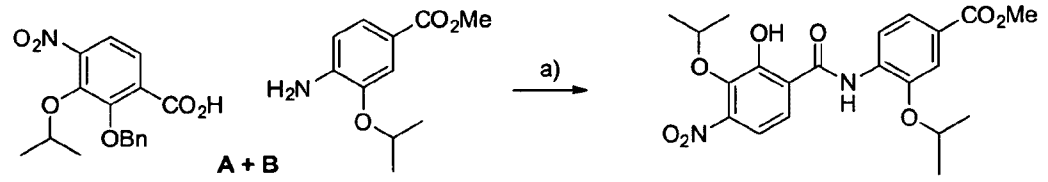
a) BBr_3, CH_2Cl_2, -40°C - rt, 17 h (95%); b) $NaBH_4$, THF,-40°C - rt, 30 min (91%); c) $PhCH(OMe)_2$, *p*TSA.H_2O, THF, rt, 5 days (56%); d) $Ni(NO_3)_2.5H_2O$, *p*TsOH.H_2O, acetone, rt, 2.5 h (74%); e) ^{i}PrOH, DEAD, PPh_3, THF, rt, 17 h (85%); f) $Pd_2(dba)_3$, $(PhO)_3P$, ^{i}PrOH, dioxane, 80 °C, 1.5 h (70%); g) Camphor-10-sulfonic acid, CH_2Cl_2/MeOH (1:2), 0 °C- rt, 17 h (90%); h) MnO_2, CH_2Cl_2, rt, 17 h (81%); i) 2-methyl-2-butene, $NaClO_2/NaH_2PO_4$, ^{t}BuOH, rt, 17 h (75%); j) $TMSCHN_2$, MeOH/PhMe, 0 °C - rt, 30 min (57%); k) BnOH, DEAD, PPh_3, THF, rt, 17 h (90%); l) LiOH, THF/H_2O (1:1), rt, 17 h (99%).

(terminal trisubstituted aromatic moiety)



a) $TMSCHN_2$, MeOH/PhMe, 0 °C - rt, 30 min (90%); b) ^{i}PrOH, DEAD, PPh_3, THF, rt, 17 h (quant.); c) Pd/C, MeOH, H_2 atm., rt, 17 h (quant.).

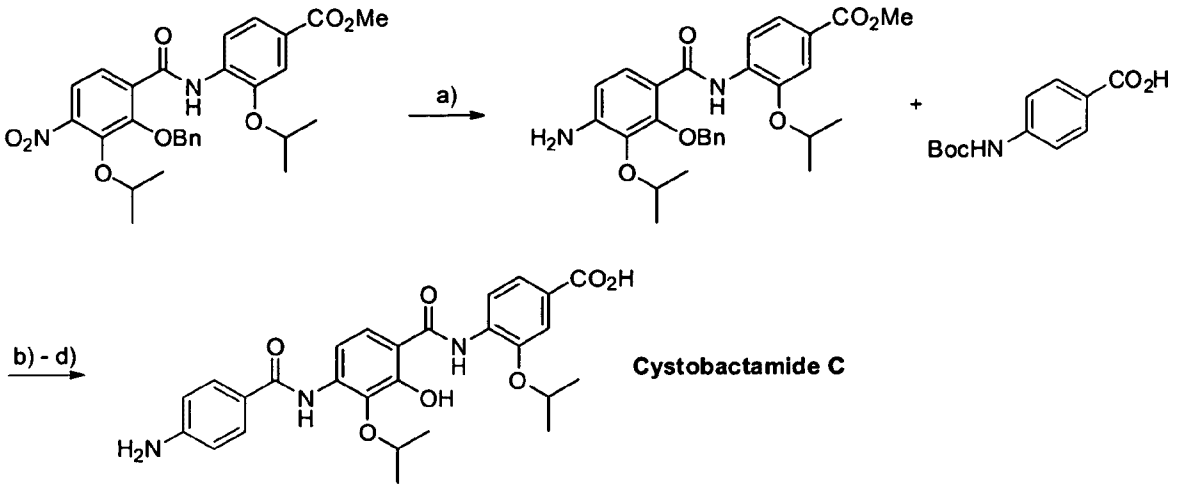
(merging aromatic moieties A and B)



a) I. **A**, Goshez´s reagent, CH_2Cl_2, 40°C, 3 h; II. **B**, DIPEA, CH_2Cl_2, rt, 10 min, then I., 40°C, 2 days (68%)

Scheme 2: Finalization of cystobactamide C synthesis.

Cystobactamide C (finalization of synthesis)



a) Pd/C, MeOH, H_2 atm., rt, 3 h (96%); b) I. 4-Boc aminobenzoic acid, Goshez's reagent, CH_2Cl_2, rt, 1 h; II. **B**, DIPEA, CH_2Cl_2; then I., rt, 1 day (72%); c) TFA/CH_2Cl_2 (10:1), rt, 17 h (quant.); d) LiOH, THF/H_2O (1:1), rt, 17 h (99%).

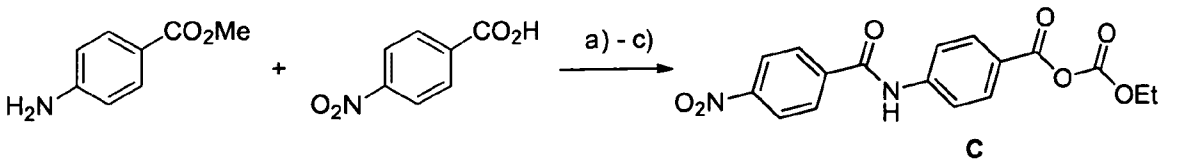
1.2 Cystobactamide A

The more complex cystobactamides consist of the bisamide that represents cystobactamide C, a bisarylamide (fragment C) and a chiral linker element. In this section fragment C and the chiral linker element are reported first which is followed by the assembling of all three elements to provide cystobactamide A.

1.2.1 Synthesis of bisarene C.

Scheme 3: Synthesis of activated fragment C.

Fragment C

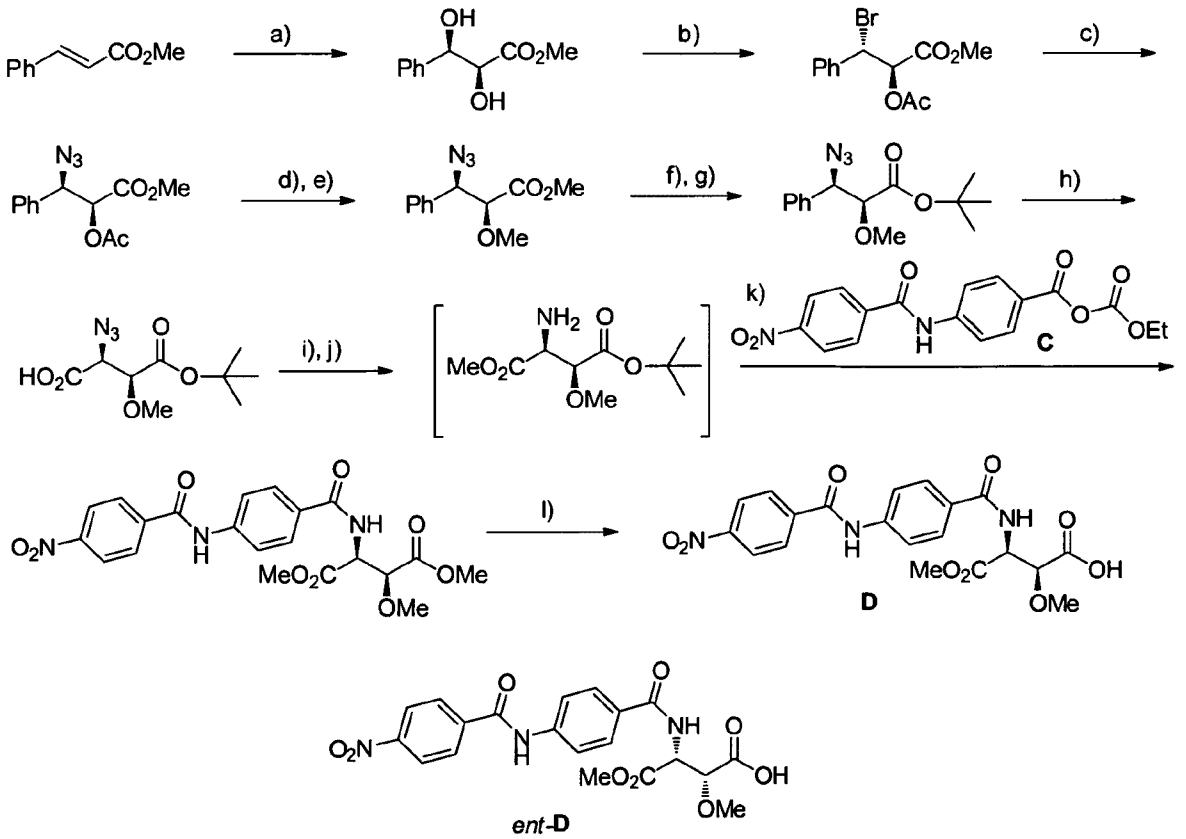


a) $P(OMe)_3$, I_2, THF, 3 days (75%); b) LiOH, THF/H_2O (1:1), rt, 17 h (80%); c) ethyl chloroformate, Et_3N, CH_3CN, 0°C 30 min (67%).

1.2.2 Synthesis of the chiral building block D with bisarene C attached

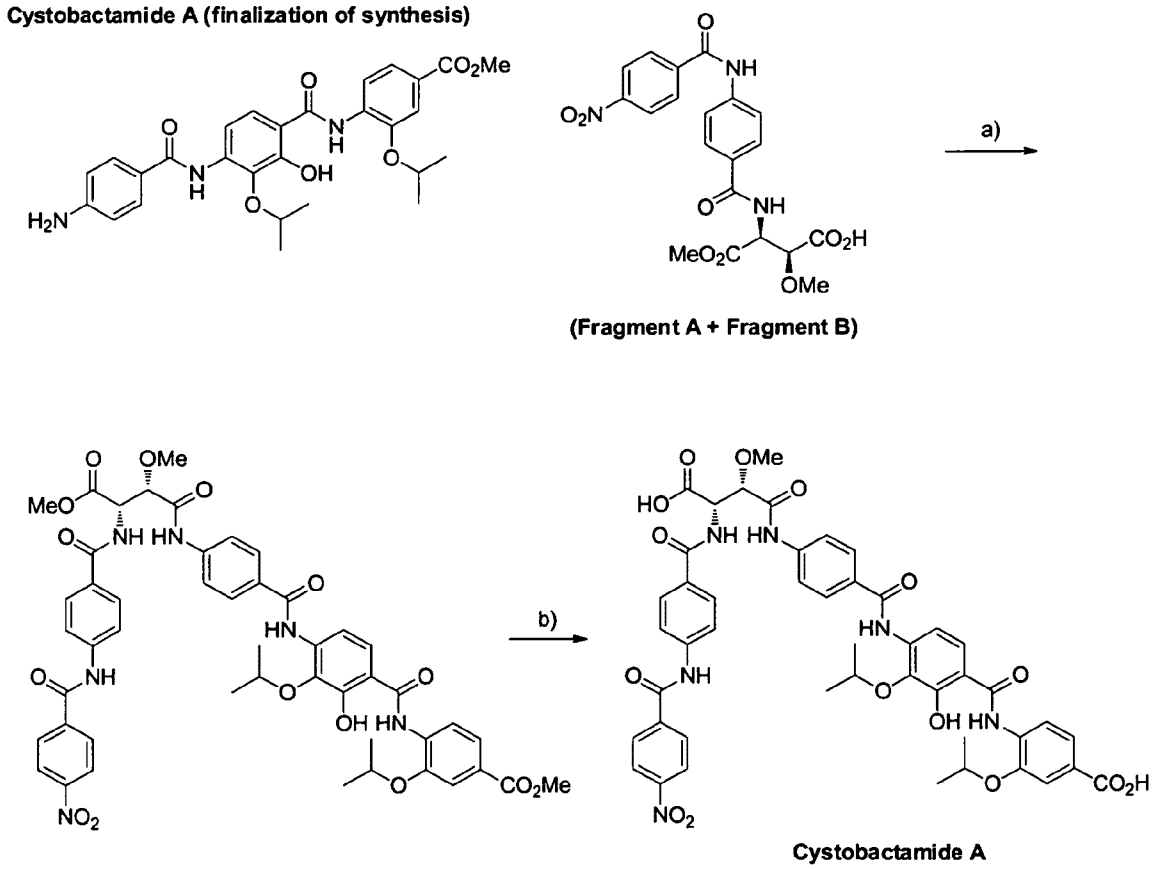
The synthesis starts from methyl cinnamate and chirality is introduced by the Sharpless asymmetric dihydroxylation. The phenyl ring serves as protecting group for the second carboxylate which is oxidatively liberated. Finally, building block C is attached to the free amino group. The corresponding enantiomeric fragment (*ent*)-D was prepared using AD mix α instead of AD mix β.

Scheme 4: Synthesis of carboxylic acid D starting from methylcinnamate.



a) AD mix β, $MeSO_2NH_2$, *t*BuOH/H_2O (1:1), 0 °C, 12 h, then 25 °C, 12 h, (79%, *ee* > 99%); b) 33% HBr/HOAc, 45 °C, 30 min.,(71%); c) NaN_3, DMF, 25 °C, 3 h, then 40 °C, 2 h, (89%); d) KOH, THF/H_2O; e) 2. MeI, Ag_2O, $CaSO_4$ (74% for two steps); f) KOH, THF/H_2O; g) Me_2N-CH(O*t*Bu)$_2$, toluene, 80 °C, (87% for two steps); h) $RuCl_3 \cdot H_2O$, $NaIO_4$, $CHCl_3/CH_3CN/H_2O$, 70 °C; i) MeI, Ag_2O, $CaSO_4$; j) Ph_3P,THF/H_2O, 50°C, k) DMF (16% for four steps); l) CF_3CO_2H, CH_2Cl_2, (quant).

Scheme 5: Finalization of cystobactamide A synthesis.



a) HOAt, EDC·HCl, DIPEA, CH_2Cl_2, rt, 17 h (75%); b) LiOH, THF/H_2O(1/1), rt, (95%).

2. Experimentals

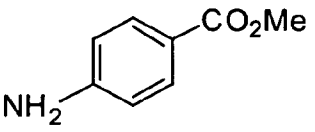
2.1 General Experimental information

All reactions were performed in oven dried glassware under an atmosphere of nitrogen gas unless otherwise stated. ^{1}H-NMR spectra were recorded at 400 MHz with a Bruker AVS-400 or at 500 MHz with a Bruker DRX-500. ^{13}C-NMR spectra were recorded at 100 MHz with a Bruker AVS-400 and at 125 MHz with a Bruker DRX-500. Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Chemical shift values of ^{1}H and ^{13}C NMR spectra are commonly reported as values in ppm relative to residual solvent signal as internal standard. The multiplicities refer to the resonances in the off-resonance decoupled spectra. These were elucidated using the distortionless enhancement by polarization transfer (DEPT) spectral editing technique, with secondary pulses at 90° and 135°. Multiplicities are reported using the following abbreviations: s = singlet (due to quaternary carbon), d = doublet (methine), t = triplet (methylene), q = quartet (methyl). Mass spectra (EI) were obtained at 70 eV with a type VG Autospec spectrometer (Micromass), with a type LCT (ESI) (Micromass) or with a type Q-TOF (Micromass) spectrometer in combination with a Waters Aquity Ultraperformance LC system. Analytical thin-layer chromatography was performed using precoated silica gel 60 F_{254} plates (Merck, Darmstadt), and the spots were visualized with UV light at 254 nm or alternatively by staining with potassium permanganate, phosphomolybdic acid, 2,4-dinitrophenol or *p*-anisaldehyde solutions. Tetrahydrofuran (THF) was distilled under nitrogen from sodium/benzophenone. Dichloromethane (CH_2Cl_2) was dried using a Solvent Purification System (SPS). Commercially available reagents were used as supplied. Preparative high performance liquid chromatography using a Merck Hitachi LaChrom system (pump L-7150, interface D-7000, diode array detector L-7450 (λ = 220-400 nm, preferred monitoring at λ = 230 nm)) with column (abbreviation referred to in the experimental part given in parentheses): Trentec Reprosil-Pur 120 C18 AQ 5 µm, 250 × 8 mm, with guard column, 40 × 8 mm (C18-SP). Flash column chromatography was performed on Merck silica gel 60 (230-400 mesh). Eluents used for flash chromatography were distilled prior to use. Melting points were measured using a

SRS OptiMelt apparatus. Optical rotations [α] were measured on a Polarimeter 341 (Perkin Elmer) at a wavelength of 589 nm and are given in 10^{-1} deg cm^2 g^{-1}.

2.2 Specific procedures

4-Aminomethylbenzoate



MeOH (200 mL) was provided in a flask and acetyl choride (2.6 mL, 36.5 mmol, 1 eq) was slowly added. Then 4-aminobenzoic acid (5.00 g, 36.5 mmol) was added and the solution was stirred 7 days at room temperature. The solvent was removed under reduced pressure and 4-aminomethylbenzoate (5.38 g, 35.59 mmol, quantitative) was obtained as a beige solid.

The titled compound decomposes before reaching its melting point.

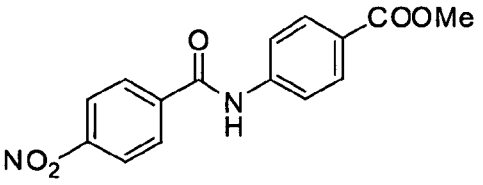
ATR-IR (neat): ü= 2828, 2015, 1724, 1612, 1558, 1508, 1430, 1316, 1280, 1181, 1109, 1072, 1022, 984, 959, 853, 786, 757, 686, 653 cm^{-1}.

^{1}H-NMR (400 MHz, CD_3OD): δ 8.19-8.13 (m, 2H), 7.53-7.48 (m, 2H), 3.93 (s, 3H) ppm.

^{13}C-NMR (100 MHz, CD_3OD): δ 167.2 137.0, 132.4, 131.7, 124.2, 53.0 ppm

HRMS (ESI): Calculated for $C_8H_{10}NO_2$ $(M+H)^+$: 152.0712, found: 152.0706.

4-(4-Nitrobenzamido)methyl benzoate



A solution of $P(OMe)_3$ (3.5 mL, 29.8 mmol) in CH_2Cl_2 (100 mL) was cooled with an ice bath, then I_2 (7.56 g, 29.8 mmol) was added. After the solid iodine was completely dissolved, *p*-nitrobenzoic acid (5.52 g, 29.8 mmol) and Et_3N (4.70 mL, 33.7 mmol)

were added in sequential order, and the solution was stirred for 10 minutes in a cooling bath. 4-aminomethylbenzoate (3.00 gr, 19.9 mmol) was added and the mixture was stirred for 10 minutes. After removing the cooling bath, the reaction mixture was stirred for 3 days at room temperature, then diluted with saturated aqueous $NaHCO_3$ and extracted with dichloromethane (3x). The combined, organic layer was sequentially washed with H_2O, 1 M HCl, H_2O, and brine. The combined organic layers were dried with anhydrous $MgSO_4$ and the solvent concentrated *in vacuo*, yielding the title compound (4.4 g, 14.65 mmol, 75%) as a beige solid.

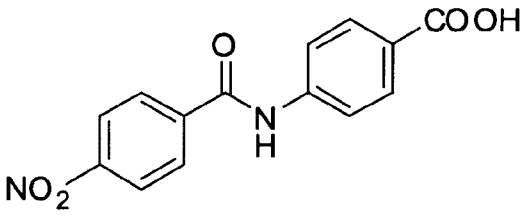
mp: 245 – 246°C

^1H NMR (400 MHz, DMSO) δ 10.87 (s, $1H_{NH}$), 8.39 (d, J = 8.8 Hz, 2H), 8.20 (d, J = 8.8 Hz, 2H), 7.99 (d, J = 8.8 Hz, 2H), 7.95 (d, J = 8.8 Hz, 2H),3.84 (s, $3H_{OMe}$) ppm.

^{13}C NMR (100 MHz, DMSO) δ 166.2, 164.9, 149.77, 143.6, 140.7, 130.7, 129.8, 125.3, 124.2, 120.2, 52.4 ppm.

HRMS (ESI): Calculated for $C_{15}H_{13}N_2O_2Na$ $(M+H)^+$: 301.0824, found: 301.0828.

4-(4-Nitrobenzamido) benzoate



4-(4-Nitrobenzamido)methyl benzoate (4.32 g, 14.38 mmol) was dissolved in a mixture 1/1 of THF/H_2O (77/77 mL). Then, solid LiOH (5.16 g, 215.66 mmol) was added and the system was stirred at room temperature for 17 hours. 1M HCl was added until pH~1 and the resulting solid was filtered and dried *in vacuo*. The title compound (3.3 g, 11.54 mmol, 80%) was obtained as a pale yellow solid.

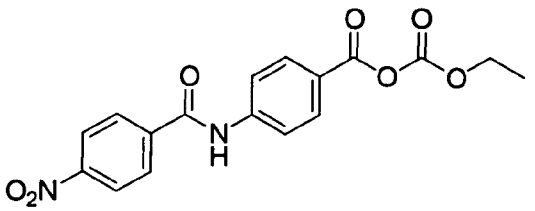
mp: 322 – 324°C

^1H NMR (400 MHz, C_6D_6) δ 10.83 (s, $1H_{CO2H}$), 8.34 (d, J = 8.6 Hz, 1H), 8.29 (d, J = 8.6 Hz, 1H), 8.13 (d, J = 8.6 Hz, 1H), 8.06 (d, J = 8.6 Hz, 1H), 7.75 (s, $1H_{NH}$) ppm.

^{13}C NMR (100 MHz, C_6D_6) δ 168.2, 164.6, 162.2, 149.7, 143.9, 141.1, 131.1, 129.8, 123.5, 120.4 ppm.

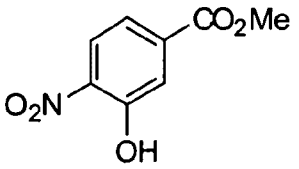
HRMS (ESI): Calculated for $C_{14}H_9N_2O_5$ $(M-H)^-$: 285.0511, found: 285.0506.

(Ethyl carbonic) 4-(4-nitrobenzamido)benzoic anhydride



To a stirred solution of 4-aminobenzoic acid (1.5 g, 10.9 mmol) and N, N-dimethylaniline (2.0 g, 10.9 mmol) in acetone was added 4-nitrobenzoyl chloride at 0°C. Then, the reaction mixture was allowed to warm to room temperature and stirred for another hour. The resulting solid was filtered and purified by recrystallization in DMF to afford 4-(4-nitro-benzoylamino)-benzoic acid (2.75g, 88%).
4-(4-Nitro-benzoylamino)-benzoic acid (0.6 g, 2.1 mmol) was dissolved in 14 ml CH_3CN. Then Et_3N (0.31ml, 2.2 mmol) was added at 0°C. To this resulting solution ethyl chloroformate was added. After stirring for 30 min at 0°C, the white precipitate was filtered and washed with cold CH_3CN, then dried under high vacuum at room temperature to afford the title anhydride 0.5 g, 67%.
^{1}H-NMR (400 MHz, DMSO, DMSO = 2.50 ppm): δ = 1.33 (dd, *J* = 7.2Hz , 3H), 4.37 (q, *J* = 7.2 Hz, 2H), 8.02-8.09 (m, 4H), 8.21(d, *J* = 8.8 Hz, 2H), 8.40 (d, *J* = 8.8 Hz, 2H), 11.01 (s, 1H).

3-Hydroxy-4-nitromethylbenzoate



$TMSCHN_2$ (2.0 M in Et_2O, 13.20 mL, 26.48 mmol) was added to a solution of 3-hydroxy-2-nitrobenzoic acid (2.50 g, 13.65 mmol) in a mixture of toluene/methanol (81/36 mL) at 0 °C. After stirring at 0 °C for 30 minutes, the solvent was evaporated

in vacuo to give an oily residue, which was purified by flash chromatography (petroleum ether/ethyl acetate= 9:1) to yield the title compound (2.43 g, 12.33 mmol, 90%) as a yellow solid.

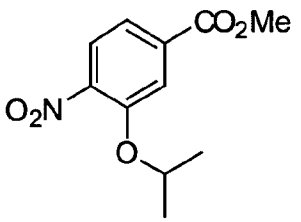
mp: 91 – 92°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.49 (s, $1H_{OH}$), 8.17 (d, *J* = 8.8 Hz, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.61 (dd, *J* = 8.8, 1.8 Hz, 1H), 3.96 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 165.0, 154.8, 138.1, 125.4, 121.8, 120.74, 53.1 ppm.

HRMS (ESI): Calculated for $C_8H_6NO_5$ $(M-H)^-$: 196.0246, found: 196.0249.

3-Isopropoxy-4-nitromethylbenzoate



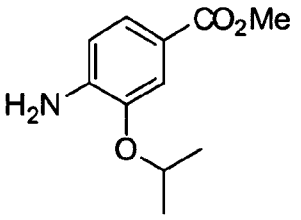
3-Hydroxy-4-nitromethylbenzoate (2.30 g, 10.89 mmol) was dissolved in THF (100 mL). ^{i}PrOH (1.10 mL, 14.16 mmol) and PPh_3 (3.90 g, 14.70 mmol) were added, and the mixture was stirred until all components were dissolved. DEAD (2.2 M in toluene, 14.16 mmol, 6.50 mL) was added and the mixture was stirred at room temperature 17 hours. The solvent was evaporated *in vacuo* to give an oily residue, which was purified by flash chromatography (petroleum ether/ethyl acetate= 95:5) to yield the title compound (2.61 g, 10.91mmol, quantitative) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.4 Hz, 2H), 7.64 (dd, *J* = 8.3, 1.6 Hz, 1H), 4.77 (hept, *J* = 6.1 Hz, 1H), 3.95 (s, 3H), 1.41 (s, 3H), 1.40 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 165.5, 150.9, 134.6, 125.2, 121.2, 117.1, 73.2, 52.9, 21.9 ppm.

HRMS (Qtof): Calculated for $C_8H_6NO_5$ $(M+Na)^+$: 262.0691, found: 262.0700.

3-Isopropoxy-4-aminomethylbenzoate



3-Isopropoxy-4-nitromethylbenzoate (2.60 g, 10.87 mmol) was dissolved in MeOH (91.0 mL) and degassed. Pd/C (10% wt., 0.58 g, 0.54 mmol) was added and vacuum was applied under cooling to remove air. The flask was flushed with H_2 and the suspension was stirred for 17 hours at room temperature. The catalyst was filtered over Celite®, washed with MeOH and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (petroleum ether/EtOAc = 7/3). 3-Isopropoxy-4-aminomethylbenzoate was obtained (2.27 g, 10.85 mmol, quantitative) as a light orange solid.

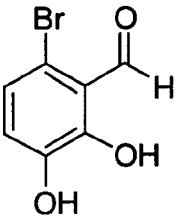
mp: 55 – 57°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.46 (d, *J* = 1.7 Hz, 1H), 6.66 (dd, *J* = 8.2, 5.1 Hz, 1H), 4.63 (sept, *J* = 5.1 Hz, 1H), 3.85 (s, 3H), 1.36 (s, 3H), 1.35 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 144.24, 142.3, 124.0, 119.5, 114.1, 113.5, 70.9, 51.8, 22.3 ppm.

HRMS (ESI): Calculated for $C_{11}H_{16}NO_3$ $(M+H)^+$: 210.1130, found: 210.1126.

6-Bromo-2,3-dihydroxybenzaldehyde



To a solution of 6-bromo-2-hydroxy-3-methoxybenzaldehyde (25.0 g, 108.2 mmol) in CH_2Cl_2 (270 mL) at -30 °C was slowly added BBr_3 (1 M in CH_2Cl_2, 200.0 mL, 200.0 mmol) via additional funnel over a period of 45 minutes. The solution was allowed to

warm to room temperature and stirred 17 hours. H_2O was added and the reaction mixture was stirred for additional 30 minutes. The solution was then extracted with EtOAc (3x) and washed with H_2O. The combined, organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo* to give the title compound (22.16 g, 102.11 mmol, 95%) as a yellow solid.

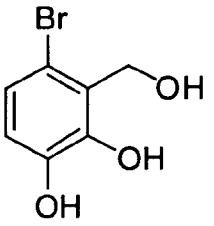
mp: 135 – 136°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 12.13 (d, *J* = 0.5 Hz, $1H_{-OH}$), 10.27 (s, $1H_{-CHO}$), 7.07 (d, *J* = 8.5 Hz, 1H), 7.02 (dd, *J* = 8.5, 0.5 Hz, 1H), 5.67 (s, $1H_{-OH}$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.4, 151.2, 145.0, 124.4, 122.0, 117.5, 116.1 ppm.

HRMS (ESI): Calculated for $C_7H_4BrO_3$ $(M-H)^-$: 214.3943, found: 214.9344.

4-Bromo-3-hydroxymethylbenzene-1,2-diol



A solution of 6-bromo-2,3-dihydroxybenzaldehyde (22.16 g, 102.10 mmol) in THF (650 mL) at -40 °C was treated with $NaBH_4$ (3.86 g, 102.10 mmol) portion wise (3x). The resulting mixture was stirred for 30 minutes at room temperature. A saturated aqueous solution of NH_4Cl was added and the mixture was stirred for another 10 minutes, before being finally treated with 1M HCl. After 10 minutes of additional stirring, the aqueous phase was extracted with EtOAc (3x). The combined, organic extracts were dried over anhydrous $MgSO_4$ and filtered .The solvent was removed under reduced pressure to yield the title compound (20.27 g, 92.53 mmol, 91%) as a colorless solid.

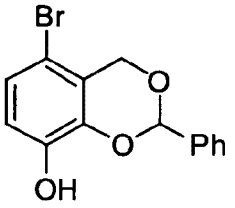
mp: 90 – 92°C

^{1}H NMR (400 MHz, MeOD) δ 6.88 (d, *J* = 8.5 Hz, 1H), 6.64 (d, *J* = 8.5 Hz, 1H), 4.82 (s, 2H) ppm.

^{13}C NMR (100 MHz, MeOD) δ 147.1, 146.1, 126.9, 123.9, 116.6, 114.4, 61.1 ppm.

HRMS (ESI): Calculated for $C_7H_6BrO_3$ $(M-H)^-$: 216.9500, found: 216.9505.

5-Bromo-2-phenyl-4*H*-benzo-[1,3]-dioxin-8-ol



A solution of 4-bromo-3-hydroxymethylbenzene-1,2-diol (20.27 g, 92.53 mmol) in THF (550 mL) was treated with $PhCH(OMe)_2$ (20.8 mL, 138.8 mmol) and *p*TSA.H_2O (0.19 g, 1.02 mmol). The mixture was stirred at room temperature for 5 days. CH_2Cl_2 was added and then washed successively with 5% aqueous $NaHCO_3$ and brine. The aqueous phase was extracted with EtOAc (3x). The combined, organic extracts were dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure. Purification by flash chromatography (petroleum ether/EtOAc = 95/5) afforded 5-bromo-2-phenyl-4*H*-benzo-[1,3]-dioxin-8-ol (16.02 g, 52.16 mmol, 56%) as a colorless solid.

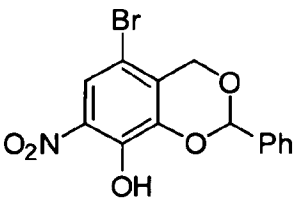
mp: 89 – 91°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 – 7.55 (m, 2H), 7.50 – 7.43 (m, 3H), 7.07 (d, *J* = 8.6 Hz, 1H), 6.78 (d, *J* = 8.6 Hz, 1H), 5.97 (s, 1H), 5.40 (s, $1H_{-OH}$), 4.99 (s, 2H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 144.0, 141.8, 136.1, 130.1, 128.8, 126.7, 124.9, 121.0, 115.0, 109.4, 100.0, 67.8 ppm.

HRMS (ESI): Calculated for $C_{14}H_{10}BrO_3$ $(M-H)^-$: 304.9813, found: 304.9813.

5-Bromo-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxin-8-ol



5-Bromo-2-phenyl-4*H*-benzo-[1,3]-dioxin-8-ol (6.00 g, 19.54 mmol; max. amount) was dissolved in acetone (250 mL). Then, $Ni(NO_3)_2.5H_2O$ (5.68 g, 19.54 mmol) and *p*TSA.H_2O (3.72 g, 19.54 mmol) were added. The mixture was stirred at room temperature for 2.5 h. The reaction mixture was filtered over Celite®, washed with CH_2Cl_2 and concentrated *in vacuo*. Purification by flash chromatography (dry load: SiO_2 + CH_2Cl_2; petroleum ether/ethyl acetate= 9:1) yielded the titel compound (5.08 g, 14.43 mmol, 74%) as a bright yellow solid.

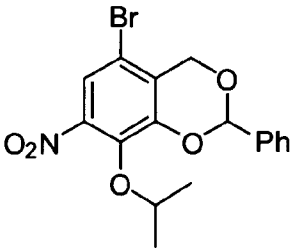
mp: 154 – 156°C

^1H NMR (400 MHz, $CDCl_3$) δ 10.60 (s, $1H_{-OH}$), 7.96 (s, 1H), 7.65 – 7.57 (m, 2H), 7.48 – 7.42 (m, 3H), 6.02 (s, 1H), 4.99 (s, 2H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 144.9, 135.5, 133.2, 130.2, 129.0, 128.9, 126.7, 119.2, 109.2, 99.9, 67.4 ppm.

HRMS (ESI): Calculated for $C_{14}H_9BrNO_5$ $(M-H)^-$: 359.9664, found: 349.9660.

5-Bromo-8-isopropoxy-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxine

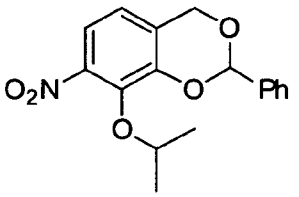


5-Bromo-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxin-8-ol (13.79 g, 39.16 mmol) was dissolved in THF (429 mL). *i*PrOH (4.00 mL, 50.91 mmol) and PPh_3 (13.87 g, 52.87 mmol) were added, and the mixture was stirred until all components were dissolved. DEAD (2.2 M in toluene, 23.1 mL, 50.91 mmol) was slowly added (via syringe pump) and the mixture was stirred at room temperature 17 hours. The solvent was evaporated *in vacuo* to give an oily residue, which was purified by flash chromatography (petroleum ether/ethyl acetate= 96:4) to yield the title compound (13. 08 g, 33.18 mmol, 85%) as a colorless solid.

mp: 87 – 89°C

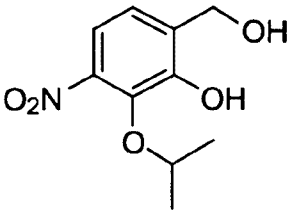
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (s, 1H), 7.59 – 7.54 (m, 2H), 7.50 – 7.43 (m, 3H), 5.97 (s, 1H), 5.00 (s, 2H), 4.69 (hept, *J* = 6.2 Hz, 1H), 1.31 (d, *J* = 6.2 Hz, 3H), 1.28 (d, *J* = 6.2 Hz, 3H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 149.0, 144.5, 139.9, 135.7, 130.1, 128.8, 126.4, 126.2, 119.8, 112.7, 99.7, 78.1, 67.6, 22.6, 22.4 ppm.
HRMS (Qtof): Calculated for $C_{14}H_9BrNO_5$ $(M+Na)^+$: 416.0110, found: 416.0101.

8-Isopropoxy-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxine , 73.



5-Bromo-8-isopropoxy-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxine 72 (4.00 g, 10.15 mmol), $Pd_2(dba)_3$ (0.93 g, 1.01 mmol), $(PhO)_3P$ (0.53 mL, 2.03 mmol), Cs_2CO_3 (4.30 g, 13.19 mmol) and ^{i}PrOH (4.7 mL, 60.88 mmol) were dissolved in 1,4-dioxane (28 mL). The oil bath was preheated to 60°C and the mixture was stirred at 80°C for 1.5 hours. The reaction mixture was filtered through Celite® and washed with EtOAc. The combined, organic extracts were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude material was purified by flash chromatography (petroleum ether/ethyl acetate= 96:4) to yield the title compound (2.24 g, 7.10 mmol, 70%) as a pale yellow solid.
mp: 80 – 82°C
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 – 7.55 (m, 2H), 7.51 – 7.41 (m, 3H), 7.37 (d, *J* = 8.5 Hz, 1H), 6.81 (d, *J* = 8.5 Hz, 1H), 6.01 (s, 1H), 5.19 (d, *J* = 15.5 Hz, 1H), 5.03 (d, *J* = 15.5 Hz, 1H), 4.71 (hept, *J* = 6.2 Hz, 1H), 1.32 (d, *J* = 6.2 Hz, 3H), 1.28 (d, *J* = 6.2 Hz, 3H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 147.67, 144.27, 140.55, 136.26, 129.85, 128.72, 126.54, 126.34, 118.82, 116.69, 99.61, 77.71, 66.44, 22.65, 22.41 ppm.
HRMS (QTof): Calculated for $C_{17}H_{17}NO_5Na$ $(M+Na)^+$: 338.1004. Found: 338.1003.

6-Hydroxymethyl-2-isopropoxy-3-nitrophenol



To a mixture of 8-isopropoxy-7-nitro-2-phenyl-4*H*-benzo-[1,3]-dioxine (4.24 g, 13.43 mmol) in MeOH (102 mL) and CH_2Cl_2 (42 mL) at 0°C was added camphor sufonic acid (3.12 g, 13.43 mmol). The mixture was stirred at room temperature for 17 hours. The reaction mixture was quenched with Et_3N until pH~8, concentrated *in vacuo* and purified by flash chromatography (petroleum ether/ethyl acetate= 7:3) to yield the title compound (2.75 g, 12.09 mmol, 90%) as a brownish solid.

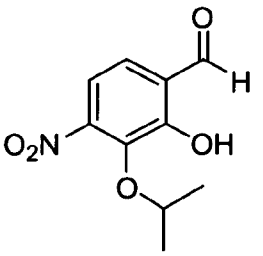
mp: 39 – 41°C

^1H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 7.4 Hz, 1H), 7.12 (d, *J* = 7.4 Hz, 1H), 6.61 (s, $1H_{-OH}$), 4.81 (d, *J* = 3.5 Hz, 2H), 4.39 (hept, *J* = 7.4 Hz, 1H), 1.36 (s, 3H), 1.35 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 148.9, 138.5, 132.4, 122.1, 116.5, 79.2, 61.3, 22.5 ppm.

HRMS (ESI): Calculated for $C_{10}H_{12}NO_5$ $(M-H)^-$: 226.0715, found: 226.0717.

2-Hydroxy-3-isopropoxy-4-nitrobenzaldehyde



6-Hydroxymethyl-2-isopropoxy-3-nitrophenol (2.97 g, 13.05 mmol) was dissolved in CH_2Cl_2 (58 mL). Then MnO_2 (11.35 g, 130.53 mmol) was added and the mixture was stirred at rt 17 h. The mixture was filtered over Celite® and washed with CH_2Cl_2. The

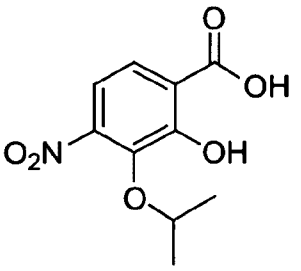
solvent was concentrated to give the title compound (2.38 g, 10.57 mmol, 81%) as a brown oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.44 (s, $1H_{-CHO}$), 9.97 (s, $1H_{-OH}$), 7.39 (d, *J* = 8.4 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 1H), 4.88 (hept, *J* = 6.2 Hz, 1H), 1.33 (s, 3H), 1.32 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.39, 156.53, 149.36, 139.74, 127.28, 122.57, 114.32, 77.42, 77.16, 22.51. ppm.

HRMS (ESI): Calculated for $C_{10}H_{10}NO_5$ $(M-H)^-$: 224.0559. Found: 224.0535.

2-Hydroxy-3-isopropoxy-4-nitrobenzoic acid



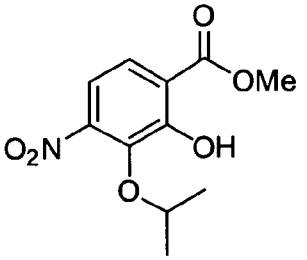
2-Hydroxy-3-isopropoxy-4-nitrobenzaldehyde (2.36 g, 10.49 mmol) was dissolved in *tert*-buthanol (71 mL). 2-Methyl-2-butene (2M in THF, 36.7 mL, 73.45 mmol) and a solution of $NaClO_2$ (2.85 g, 31.48 mmol) and NaH_2PO_4 (6.32 g, 47.22 mmol) in H_2O (51 mL) were added in sequential order. The reaction mixture was stirred at room temperature for 17 hours. 6M NaOH was added until ph~10 and the solvent was concentrated *in vacuo*. H_2O was added and the organic layer was extracted with petroleum ether (2x). The aqueous layer was acidified with 6M HCl until pH~1 and extracted with ethyl acetate (3x). The organic extracts were combined, dried over $MgSO_4$ and filtered. The solvent was concentrated *in vacuo* to yield the title compound (1.90 g, 7.87 mmol, 75%) as a dark wax.

^{1}H NMR (400 MHz, MeOD) δ 7.72 (d, *J* = 8.7 Hz, 1H), 7.15 (d, *J* = 8.7 Hz, 1H), 4.86 – 4.82 (m, 1H), 1.28 (s, 3H), 1.26 (s, 3H) ppm.

^{13}C NMR (100 MHz, MeOD) δ 172.7, 158.0, 140.0, 125.8, 117.4, 113.8, 77.5, 22.6 ppm.

HRMS (ESI): Calculated for $C_{10}H_{10}NO_6$ $(M-H)^-$: 240.0508, found: 240.0510.

2-Hydroxy-3-isopropoxy-4-nitrobenzoate



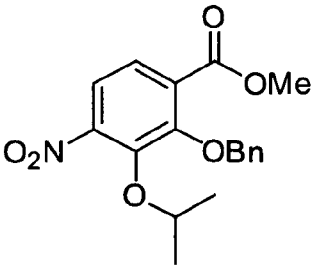
$TMSCHN_2$ (2.0 M in Et_2O, 0.87 mL, 1.75 mmol) was added to a solution of 2-hydroxy-3-isopropoxy-4-nitrobenzoic acid (0.32 g, 1.35 mmol) in a mixture of toluene/methanol (10.4/2 mL) at 0 °C. After stirring at 0 °C for 30 minutes, the solvent was evaporated *in vacuo* to give an oily residue, which was purified by flash chromatography (SiO_2.Et_3N; petroleum ether/ethyl acetate= 95:5) to yield the title compound (0.24 g, 0.94 mmol, 57%) as a yellow oil.

^1H NMR (400 MHz, $CDCl_3$) δ 11.29 (s, $1H_{OH}$), 7.63 (d, J = 8.8 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H), 4.84 (hept, J = 6.2 Hz, 1H), 4.00 (s, 3H), 1.32 (s, 3H), 1.31 (s, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.2, 188.9, 176.1, 170.0, 157.0, 149.2, 139.8, 123.9, 115.7, 113.4, 77.4, 53.2, 22.5 ppm.

HRMS (ESI): Calculated for $C_{11}H_{12}NO_6$ $(M-H)^-$: 254.0665, found: 254.0666.

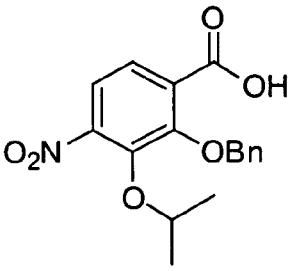
2-Benzyloxy-3-isopropoxy-4-nitrobenzoate



2-Hydroxy-3-isopropoxy-4-nitrobenzoate (0.17 g, 0.69 mmol) was dissolved in THF (7.5 mL). BnOH (92.6 µL, 0.89 mmol) and PPh_3 (0.24 g, 0.93 mmol) were added, and the mixture was stirred until all components are dissoved. DEAD (2.2 M in toluene, 0.41 mL, 0.89 mmol) was slowly added (via syringe pump) and the mixture was

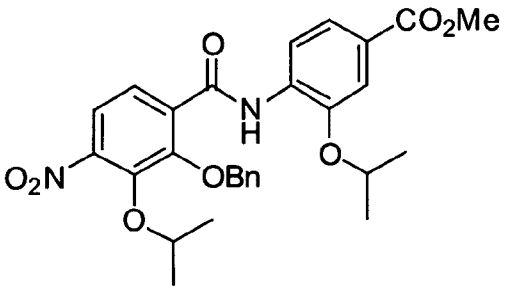
stirred at room temperature 17 hours. The solvent was evaporated *in vacuo* to give an oily residue, which was purified by flash chromatography (petroleum ether/ethyl acetate= 95:5) to yield the title compound (0.20 g, 0.58 mmol, 85%) as a colorless oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.6 Hz, 1H), 7.50 (d, *J* = 8.6 Hz, 1H), 7.48 – 7.44 (m, 2H), 7.42 – 7.35 (m, 3H), 5.14 (s, 2H), 4.74 (hept, *J* = 6.2 Hz, 1H), 3.86 (s, 3H), 1.28 (s, 3H), 1.26 (s, 3H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3, 153.4, 148.4, 145.7, 136.4, 130.9, 128.7, 128.7, 128.7, 125.1, 119.3, 78.2, 76.4, 52.8, 22.5 ppm.
HRMS (QTof): Calculated for $C_{18}H_{19}NO_6Na$ $(M+Na)^+$: 368.1110, found: 368.1112.

2-Benzyloxy-3-isopropoxy-4-nitrobenzoic acid



2-Benzyloxy-3-isopropoxy-4-nitrobenzoate (0.23 g, 0.67 mmol) was dissolved in a mixture 1/1 of THF/H_2O (3.5/3.5 mL). Then, solid LiOH (0.16 g, 6.67 mmol) was added and the reaction mixture was stirred at room temperature for 17 hours. The aqueous layer was acidified with 1M HCl until pH~1 and extracted with EtOAc (3x). The organic extracts were combined, dried over anhydrous $MgSO_4$ and filtered. The solvent was concentrated *in vacuo* to yield the title compound (0.21 g, 0.63 mmol, 95%) as a yellow wax.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 8.7 Hz, 1H), 7.58 (d, *J* = 8.7 Hz, 1H), 7.41 (s, 5H), 5.35 (s, 2H), 4.71 – 4.62 (m, 1H), 1.36 (s, 3H), 1.35 (s, 3H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 164.3, 152.8, 149.7, 144.7, 134.1, 129.8, 129.4, 129.2, 126.98, 120.0, 79.1, 77.7, 22.5 ppm.
HRMS (ESI): Calculated for $C_{17}H_{16}NO_6$ $(M-H)^-$: 330.0978, found: 330.0976.

4-(2-(Benzyloxy)-3-isopropoxy-4-nitrobenzamido)-3-isopropoxybenzoate



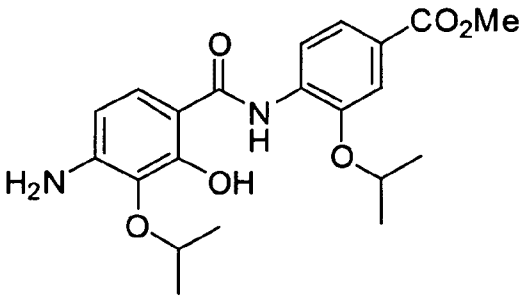
2-Benzyloxy-3-isopropoxy-4-nitrobenzoic acid (51.5 mg, 0.16 mmol) was dissolved in CH_2Cl_2 (8 mL) and preactivated with Ghosez's reagent (66.0 µL, 0.50 mmol) for 3 hours at 40 °C. 3-Isopropoxy-4-aminomethylbenzoate (0.12 g, 0.55 mmol) was dissolved in CH_2Cl_2 (8 mL) and *N,N*-diisopropylethylamine (DIPEA) was added (0.20 mL, 1.12 mmol). The solution containing the acid chloride was then added and the reaction mixture stirred for 2 days at 40 °C. The solvent was then removed and the crude product was purified by preparative HPLC (RP-18; run time 100 min; H_2O/MeCN = 100 : 0 →0 : 100; tr = 80 min) providing the title compound (56.9 mg, 0.11 mmol, 68%) as a light yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.33 (s, 1H$_{NH}$), 8.55 (d, *J* = 8.5 Hz, 1H), 7.85 (d, *J* = 8.7 Hz, 1H), 7.70 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.59 (d, *J* = 8.7 Hz, 1H), 7.57 (d, *J* = 1.7 Hz, 1H), 7.25 – 7.12 (m, 5H), 5.25 (s, 2H), 4.75 – 4.67 (m, 1H), 4.67 – 4.59 (m, 1H), 3.93 (s, 3H), 1.40 (d, *J* = 6.2 Hz, 6H), 1.28 (d, *J* = 6.0 Hz, 6H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 167.0, 161.4, 151.1, 147.9, 146.1, 145.2, 134.1, 132.9, 132.9, 130.0, 129.4, 128.7, 125.79, 125.6, 123.3, 120.1, 119.5, 113.3, 78.9, 77.4, 71.7, 52.3, 22.6, 22.1 ppm.

HRMS (ESI): Calculated for $C_{28}H_{31}N_2O_8$ $(M+H)^+$: 523.2080, found: 523.2075.

4-(4-Amino-2-hydroxy-3-isopropoxybenzamido)-3-isopropoxybenzoate



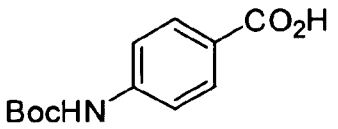
4-[2-(Benzyloxy)-3-isopropoxy-4-nitrobenzamido]-3-isopropoxy-benzoate (7.9 mg, 0.015 mmol) was dissolved in MeOH (0.5 mL) and degassed. Pd/C (10% wt., 2 mg, 0.0014 mmol) was added and vacuum was applied under cooling to remove air. The flask was flushed with H_2 and the suspension was stirred for 3 hours at room temperature. The catalyst was filtered off over Celite®, washed with MeOH and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (petroleum ether/ethyl acetate= 7:3) and the title compound was obtained (5.8 g, 0.014 mmol, 96%) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 12.21 (s, $1H_{-OH}$), 8.81 (s, $1H_{-NH}$), 8.49 (d, J = 8.5 Hz, 1H), 7.69 (dd, J = 8.5, 1.8 Hz, 1H), 7.58 (d, J = 1.7 Hz, 1H), 7.07 (d, J = 8.8 Hz, 1H), 6.28 (d, J = 8.7 Hz, 1H), 4.80 – 4.72 (m, 1H), 4.72 – 4.63 (m, 1H), 4.28 (s, $2H_{-NH2}$), 3.91 (s, 3H), 1.44 (d, J = 6.1 Hz, 6H), 1.34 (d, J = 6.2 Hz, 7H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 168.5, 166.9, 156.4, 146.5, 146.0, 132.7, 132.0, 125.1, 123.40, 121.5, 119.1, 113.4, 106.5, 106.3, 77.4, 74.4, 72.0, 52.3, 22.9, 22.4 ppm.

HRMS (ESI): Calculated for $C_{21}H_{25}N_2O_6$ $(M-H)^-$: 401.1713, found: 401.1716.

4-(*tert*-butoxycarbonylamino)benzoic acid

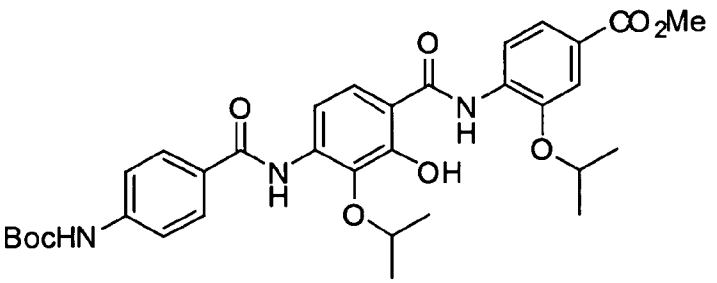


4-Aminobenzoic acid (1.00 g, 7.29 mmol) was dissolved in 1,4-dioxane (15 mL) and H_2O (7 mL). Et_3N (2.0 mL, 14.58 mmol) was added to the solution and the reaction mixture was stirred for 5 minutes at room temperature. Di-*tert*-butyl dicarbonate (3.18 g, 14.58 m mol) was then added to the solution in one portion and the reaction mixture was stirred for 24 hours. Following removal of the solvent *in vacuo*, 3M HCl was added to the residue yielding a white precipitate. The slurry was then filtered and washed with H_2O before drying in under high vacuum. Recrystallization from hot methanol yielded the titled compound as a colorless solid (1.63 g, 6.85 mmol, 94 % yield).

mp: 192 – 194 °C.

^{1}H NMR (400 MHz, DMSO) δ 9.73 (s, 1H$_{CO2H}$), 7.83 (d, 2H, *J*= 8.9 Hz), 7.55 (d, 2H, *J*=8.9 Hz), 1.47 (s, 9H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 152.6, 143.8, 130.4, 124.0, 117.2, 79.7, 28.1 ppm.
HRMS (ESI): Calculated for $C_{12}H_{15}NnaO_4$ $(M+Na)^+$: 260.0893, found: 260.0897.
The spectroscopic data are in accordance with those reported in the literature (*J.Am.Chem.Soc.* **2012**, *134*, 7406–7413).

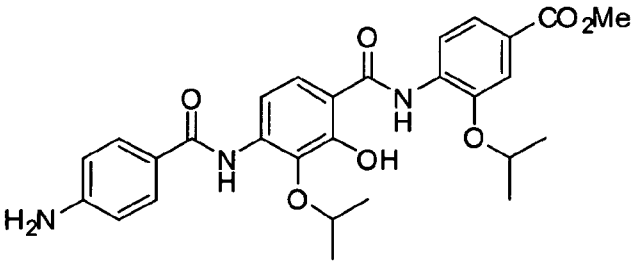
Methyl-4-(4-(4-(*tert*-butoxycarbonyl)amino)benzamido)-2-hydroxy-3-isopropxybenzamido)-3-isopropoxybenzoate



4-(*Tert*-butoxycarbonylamino)benzoic acid (40.0 mg, 0.17 mmol) was dissolved in CH_2Cl_2 (8.4 mL) and preactivated with Ghosez's reagent (22.5 µL, 0.17 mmol) for 2 hours at room temperature. 4-(4-Amino-2-hydroxy-3-isopropoxybenzamido)-3-isopropoxybenzoate (68.4 mg, 0.17 mmol) was dissolved in CH_2Cl_2 (8.4 mL) and *N*,*N*-diisopropylethylamine (DIPEA) was added (59.2 µL, 0.34 mmol). The solution containing the acid chloride was then added and the reaction mixture stirred for 1 day at room temperaturet. The solvent was then removed and the crude product was purified by preparative HPLC (RP-18; run time 100 min; H_2O/MeCN = 100 : 0 →0 : 100; tr = 70 min) providing the title compound as a light yellow oil (47.3 mg, 0.076 mmol, 72%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, *J* = 7.5 Hz, 2H), 7.78 (d, *J* = 1.4 Hz, 1H), 7.72 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.69 (s, 1H$_{NH}$), 7.68 (d, *J* = 7.3 Hz, 3H), 7.56 (d, *J* = 7.5 Hz, 1H), 7.17 (d, *J* = 7.5 Hz, 1H), 5.72 (s, 1H$_{NH}$), 5.49 (s, 1H$_{NH}$), 4.02 – 3.96 (m, 2H), 3.95 (d, *J* = 3.7 Hz, 3H), 1.49 (s, 9H), 1.46 (d, *J* = 5.6 Hz, 6H), 1.41 (d, *J* = 5.5 Hz, 6H) ppm.

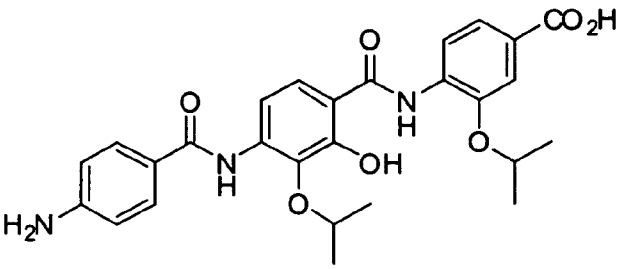
^{13}C NMR (100 MHz, $CDCl_3$) δ 166.89, 166.67, 166.61, 158.88, 154.93, 146.90, 141.47, 135.07, 134.68, 131.70, 130.38, 130.38, 127.26, 127.17, 123.25, 121.40, 120.63, 120.63, 115.87, 114.85, 113.39, 106.06, 80.65, 75.89, 74.13, 52.08, 28.41, 28.41, 28.41, 21.80, 21.80, 21.80, 21.80 ppm.
HRMS (ESI): Calculated for $C_{33}H_{38}N_3O_9$ $(M-H)^-$: 620.2687, found: 620.2689.

Methyl-4-(4-(4-aminobenzamido)-2-hydroxy-3-isopropxybenzamido)-3-isopropoxybenzoate



Methyl-4-(4-(4-(*tert*-butoxycarbonyl)amino)benzamido)-2-hydroxy-3-isopropxybenzamido)-3-isopropoxybenzoate (40.0 mg, 0.064 mmol) was dissolved in a mixture 10/1 dichloromethane/trifluoroacetic acid (1 mL) and stirred 17 hours at room temperature. The solvent was removed under reduced pressure and the residual acid was removed under high vacuum to give the titled compound (33.4 mg, 0.064 mmol, quantitative) as yellow oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (d, *J* = 1.4 Hz, 1H), 7.83 (s, $1H_{-NH}$), 7.79 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.75 (d, *J* = 7.5 Hz, 1H), 7.70 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 1H), 7.05 (d, *J* = 7.5 Hz, 1H), 6.94 (s, $1H_{-NH}$), 6.75 (d, *J* = 7.5 Hz, 2H), 6.09 (s, $1H_{-OH}$), 4.02 – 3.97 (m, 1H), 3.95 – 3.89 (s, 3H), 3.92 (m, 1H), 3.85 (s, $2H_{-NH}$), 1.47 (d, *J* = 5.7 Hz, 6H), 1.40 (d, *J* = 5.5 Hz, 6H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$) δ 166.89, 166.67, 166.61, 158.88, 152.59, 146.90, 135.07, 134.68, 131.70, 130.93, 130.93, 127.17, 123.25, 122.42, 121.40, 115.87, 114.85, 114.35, 114.35, 113.39, 106.06, 75.89, 74.13, 52.08, 21.80, 21.80, 21.80, 21.80 ppm.
HRMS (ESI): Calculated for $C_{28}H_{32}N_3O_7$ $(M+H)^+$: 522.2162, found: 522.2160.

Cystobactamide C



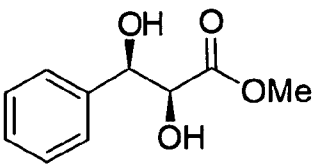
Methyl-4-[4-(4-aminobenzamido)-2-hydroxy-3-isopropxybenzamido]-3-isopropoxybenzoate (30.0 mg, 0.058 mmol) was dissolved in a mixture 1/1 of THF/H_2O (0.3/0.3 mL). Then, solid LiOH (13.9 mg, 0.58 mmol) was added and the reaction mixture was stirred at room temperature for 17 hours. The aqueous layer was acidified with 1M HCl until pH~1 and extracted with ethyl acetate (3x). The organic extracts were combined, dried over anhydrous $MgSO_4$ and filtered. The solvent was concentrated *in vacuo* to yield the title compound (27.4 mg, 0.054 mmol, 93%) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 1.4 Hz, 1H), 7.87 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.70 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 1H), 7.53 (d, *J* = 7.5 Hz, 1H), 7.05 (d, *J* = 7.5 Hz, 1H), 6.95 (s, $1H_{-NH}$), 6.77 (s, $1H_{-NH}$), 6.75 (d, *J* = 7.5 Hz, 2H), 6.12 (s, $1H_{-OH}$), 3.97 – 3.89 (m, 2H), 3.85 (s, $2H_{-NH}$), 1.40 (d, *J* = 5.5 Hz, 6H), 1.39 (d, *J* = 5.5 Hz, 6H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 167.79, 166.67, 166.61, 158.88, 152.59, 149.81, 136.38, 135.07, 134.68, 130.93, 130.93, 125.08, 123.25, 122.80, 122.42, 120.37, 114.35, 114.35, 113.76, 113.39, 106.06, 75.89, 74.13, 21.80, 21.80, 21.80, 21.80 ppm.

HRMS (ESI): Calculated for $C_{28}H_{32}N_3O_7$ $(M+H)^+$: 508.2006, found: 508.2008.

(2*S*,3*R*)-Methyl 2,3-dihydroxy-3-phenylpropanoate



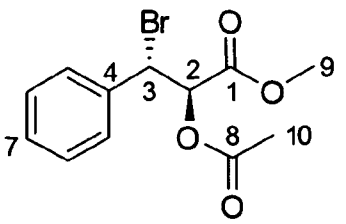
AD mix β (20.0 g) was dissolved in a mixture of *t*BuOH/H_2O (1:1; 142 mL) at 25 °C. Afterwards, $CH_3SO_2NH_2$ (1.36 g, 14.3 mmol, 1.0 eq.) was added and the reaction mixture cooled to 0 °C. Then, methylcinnamate (2.31 g, 14.3 mmol, 1.0 eq.) was added and the resulting mixture was vigorously stirred for 16 h at 0 °C. Stirring was continued for additional 6 h at 25 °C. The reaction mixture was hydrolyzed by addition of an aqueous Na_2SO_3 solution (21.4 g, 170 mmol, 12.0 eq.) and stirring was continued for additional 2.5 h. The reaction mixture was diluted with ethyl acetate and the layers were separated. The aqueous layer was extracted with EtOAc (3x). The combined organic layers were washed with H_2O (1x) and dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography (petroleum ether/ethyl acetate= 1:1) afforded the desired diol (2.21 g, 11.3 mmol, 79%) as a colorless solid. The spectroscopic data are in accordance with those reported in the literature.

R_f = 0.38 (PE/EtOAc 1:1); m.p.= 84 – 85 °C (lit: m.p.= 80 – 81 °C); $[\alpha]_D^{20}$ = -9.8° (*c* 1.28, $CHCl_3$) {lit.: $[\alpha]_D^{26}$= -9.8° (*c* 1.07, $CHCl_3$)};

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 7.42-7.29 (5H, m, ArH), 5.03 (1H, dd, *J* = 2.7, 7.2 Hz, H-3), 4.38 (1H, dd, *J* = 2.7, 6.0 Hz, H-2), 3.82 (3H, s, H-8), 3.12 (1H, d, *J* = 6.0 Hz, OH-□), 2.76 (1H, d, *J* = 7.2 Hz, OH-β) ppm;

^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.16 ppm): δ = 173.3 (q, C-1), 140.1 (q, C-4), 128.6 (2C, t, C-6), 128.3 (t, C-7), 126.3 (2C, t, C-5), 74.8 (t, C-2), 74.6 (t, C-3), 53.1 (p, C-8) ppm; HRMS (ESI): *m/z* calculated for $C_{10}H_{12}O_4Na$ $[M + Na]^+$: 219.0633; found 219.0633.

(2*R*,3*S*)-Methyl 2-acetoxy-3-bromo-3-phenylpropanoate (3)



To (2*S*,3*R*)-Methyl 2,3-dihydroxy-3-phenylpropanoate (2.15 g, 10.9 mmol, 1.0 eq.) was added HBr/HOAc (33%; 16.9 mL) dropwise at 25 °C. The resulting mixture was heated to 45 °C and stirred for 30 min. Then, the reaction mixture was cooled to 25 °C and poured into an ice-cooled $NaHCO_3$-solution (40 mL). The aqueous layer was extracted with Et_2O (3x). The combined organic layers were washed with H_2O (1x) and with brine. Then, the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography (petroleum ether/ethyl acetate= 12.5:1) gave the title compound (2.32 g, 7.71 mmol, 71%) as a colorless solid. The spectroscopic data are in accordance with those reported in the literature.

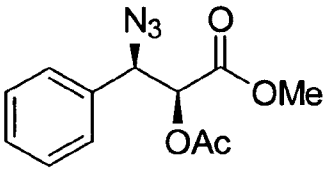
R_f = 0.79 (PE/EtOAc 1:1); m.p.= 78 – 82 °C (lit: m.p. =78 – 79 °C); $[\alpha]_D^{20}$ = +89.9° (*c* 1.74, $CHCl_3$) {Lit.: $[\alpha]_D^{26}$ = +100.3° (*c* 1.36, $CHCl_3$)};

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 7.46-7.44 (2H, m, H-6), 7.36-7.30 (3H, m, H-5, H-7), 5.65 (1H, d, *J* = 6.3 Hz, H-3), 5.35 (1H, d, *J* = 6.3 Hz, H-2), 3.71 (3H, s, H-9), 2.11 (3H, s, H-10) ppm;

^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.16 ppm): δ = 169.7 (q, C-1), 167.5 (q, C-8), 136.8 (q, C-4), 129.3 (t, C-7), 128.7 (4C, t, C-5, C-6), 75.4 (t, C-3), 52.9 (p, C-9), 49.3 (t, C-2), 20.6 (p, C-10) ppm;

HRMS (ESI): *m/z* calculated for $C_{12}H_{13}O_4BrNa$ $[M + Na]^+$: 322.9895; found 322.9891.

(2*S*,3*R*)-Methyl 2-acetoxy-3-azido-3-phenylpropanoate



(*2S,3R*)-Methyl 2-acetoxy-3-azido-3-phenylpropanoate (2.27 g, 7.55 mmol, 1.0 eq.) was dissolved in DMF (27.0 mL) at 25 °C. Then, NaN_3 (1.96 g, 30.2 mmol, 4.0 eq.) was added and the resulting mixture was heated up to 40 °C for 3 h. After cooling the reaction mixture was cooled to 25 °C and EtOAc was added. The organic layer was washed with H_2O (2x), followed by brine (1x). The combined, organic phases were

dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography (petroleum ether/ ethyl acetate= 10:1) afforded the title compound (1.77 g, 6.71 mmol, 89%) as yellow oil. The spectroscopic data are in accordance with those reported in the literature.

R_f = 0.24 (PE/EtOAc= 10:1); $[\alpha]_D^{20}$ = -97.8° (*c* 2.3, $CHCl_3$); {lit.: $[\alpha]_D^{26}$ = -104.2° (*c* 2.33, $CHCl_3$)};

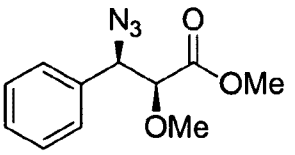
IR: $\tilde{\nu}$ = 2955 (w), 2103 (s, azide), 1747 (s, C=O), 1495 (w), 1454 (m), 1437 (m), 1373 (m), 1210 (s), 1099 (m), 1030 (m), 910 (m), 751 (m), 701 (s) cm^{-1};

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 7.42-7.33 (5H, m, ArH), 5.24 (1H, d, *J* = 4.8 Hz, H-2), 5.07 (1H, d, *J* = 4.8 Hz, H-3), 3.69 (3H, s, H-9), 2.14 (3H, s, H-10) ppm;

^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.16 ppm): δ = 169.9 (q, C-1), 168.0 (q, C-8), 134.6 (q, C-4), 129.3 (t, C-7), 129.0 (2C, t, C-6), 127.6 (2C, t, C-5), 74.9 (t, C-2), 65.4 (t, C-3), 52.8 (p, C-9), 20.5 (p, C-10) ppm;

HRMS (ESI): *m/z* calculated for $C_{12}H_{13}N_3O_4Na$ $[M + Na]^+$: 286.0804; found 286.0805.

(2*S*,3*R*)-Methyl 3-azido-2-methoxy-3-phenylpropanoate



(2*S*,3*R*)-Methyl 2-acetoxy-3-azido-3-phenylpropanoate (2.5g, 1.0 eq) was dissolved in 190 ml THF at 0 °C. Then a solution of KOH (0.5M, 10.0eq) was added dropwise and the reaction mixture was stirred at 0 °C for 5h. Afterwards, aqueous 2N HCl was added to the reaction mixture and the aqueous phase was extracted with ethyl acetate. The organic phases were combined and dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude acid which was directly used for the next step without further purification. The crude material (0.5 g, 1.0 eq) was dissolved in 17 ml methyl iodide. Then, $CaSO_4$ (2.6 g, 8.0 eq) and Ag_2O (1.7 g, 3.0 eq) were added and stirring of the suspension was carried out in the dark at room

temperature for 22 h. Then, the crude mixture was filtered and concentrated in vacuum to give the title compound (70% yield) which can be directly used in the next step without further purification.

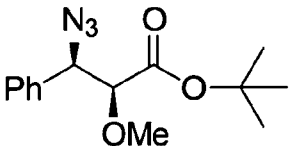
$[\alpha]_D^{20}$ = -143.7° (*c* 1.1, $CHCl_3$);

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 3.44 (s, 3H), 3.61 (s , 3H), 3.94 (d, *J* = 6.4Hz , 1H), 4.79 (d , *J* = 6.4Hz , 1H), 7.35-7.36 (m, 5H);

^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.0 ppm): δ = 52.2, 59.1, 66.9, 84.7, 127.7, 128.7, 128.9, 135.1, 170.0;

HRMS (ESI): *m/z* calculated for $C_{11}H_{13}N_3O_3Na$ $[M + Na]^+$: 258.0855; found 258.0852.

(*2S,3S*)-*tert*-Butyl 3-azido-2-methoxy-3-phenylpropanoate



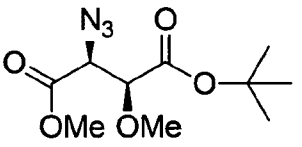
To a stirred solution of (*2S,3R*)-Methyl 3-azido-2-methoxy-3-phenylpropanoate (1.2g, 1.0 eq) in 100 ml THF was added an aqueous solution of KOH (0.5 M, 10.0 eq) dropwise. The reaction mixture was stirred for 5h at rt and hydrolyzed by addition of 2N HCl. The aqueous phase was extracted with ethyl acetate and the combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure to give carboxylic acid (1.2g, 98% yield) which was subjected to the next reaction without further purification. Crude acid (0.3g, 1.0 eq) and 3.9 ml dimethylformamide di-tert-butyl acetal (3.9 ml,12 eq) were dissolved in 8 ml toluene at room temperature. The resulting reaction mixture was heated up to 80 °C and stirred for 7h. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (petroleum ether/ethyl acetate= 30:1) to afford the title compound (0.34 g, 89% yield).

$[\alpha]_D^{20}$ = -113.3 ° (*c* 1.0, $CHCl_3$);

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$= 7.26 ppm): δ = 1.26 (s, 9H), 3.45 (s , 3H), 3.85 (d, *J* = 7.2Hz , 1H), 4.70 (d, *J* = 7.2Hz , 1H), 7.34-7.35 (m, 5H);

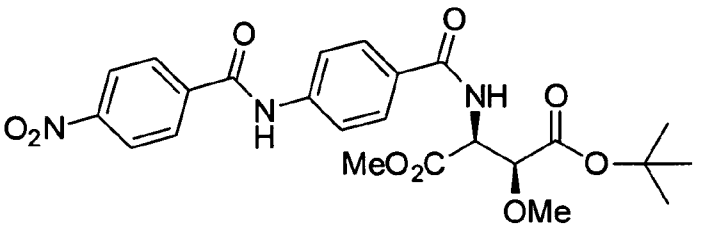
^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$= 77.0 ppm): δ = 27.7, 58.6, 67.2, 82.3, 85.1, 128.2, 128.6, 128.9, 135.2, 168.5;
HRMS (ESI): *m/z* calculated for $C_{14}H_{19}O_3N_3Na$ $[M + Na]^+$: 300.1324; found 300.1332.

(*2S,3S*)-4-*tert*-Butyl 1-methyl 2-azido-3-methoxysuccinate



To a stirred solution of (*2S,3S*)-*tert*-butyl 3-azido-2-methoxy-3-phenylpropanoate (310 mg, 1.0 eq) in a solvent mixture of 3 ml $CHCl_3$, 13 ml CH_3CN and 26 ml H_2O $NaIO_4$ (7.2 g, 30 eq) and $RuCl_3$ (0.3 eq, 69 mg) were added portionwise at room temperature. The reaction mixture was heated under refluxing conditions for 3h. A white precipitate formed upon cooling to room temperature. The solid was filtered off and the filtrate was extracted with diethyl ether. The combined organic phases were concentrated under reduced pressure to yield the crude product. This material was dissolved in 9 ml methyl iodide. Then, $CaSO_4$ (1.2 g, 8.0 eq) and Ag_2O (778 mg, 3.0 eq) were added and the reaction mixture was stirred in the dark at room temperature for 22 h. After filtration the filtrate was concentrated under reduced pressure to afford the title compound in pure form so that it can directly be employed in the next step without further purification.
^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 1.51 (s, 3H), 3.48 (s, 3H), 4.15 (d , *J* = 3.6Hz , 1H), 4.21 (d, *J* = 4.0 Hz , 1H);
^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.0 ppm): δ = 28.1, 53.0, 59.5, 63.4, 81.2, 83.0, 167.7, 168.3.

(*2S,3R*)-1-*tert*-Butyl 4-methyl 2-methoxy-3-[4-(4-nitrobenzamido)benzamido]succinate



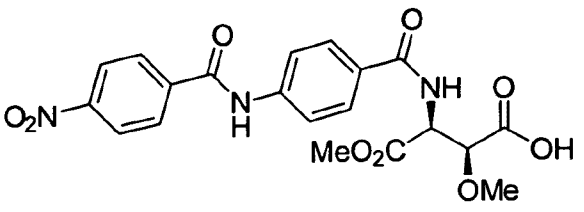
The crude mixture (*2S,3S*)-4-*tert*-butyl 1-methyl 2-azido-3-methoxysuccinate was dissolved in 12 ml THF, then 0.5 ml water and PPh_3 (881mg, 3.0eq) were added. The resulting reaction mixture was warmed up to 50 °C and stirring was continued for 12 hours. Then, the solvent was removed under reduced pressure to afford the crude product which was pure enough to be used directly in the next step. The crude product was dissolved in 5 ml DMF and (ethyl carbonic) 4-(4-nitrobenzamido)benzoic anhydride (481 mg, 1.2 eq) was added at room temperature. After stirring for 20 h, water was added and the aqueous solution was extracted with ethyl acetate. The combined organic phases were concentrated under reduced pressure. Purification by flash column chromatography (petroleum ether/ ethyl acetate= 2:1) afforded the title compound (81 mg, 16% over four steps).

$[\alpha]_D^{20}$ = -11.8 ° (*c* 1.1, $CHCl_3$);

^{1}H-NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26 ppm): δ = 1.41 (s, 9H), 3.45 (s, 3H), 3.78 (s, 3H), 4.34 (d, *J* = 2.4Hz , 1H), 5.29 (dd, *J* = 2.4, 9.6 Hz, 1H), 6.76 (d, *J* = 9.6Hz , 1H), 7.27-7.35 (m, 4H), 8.07 (d, *J* = 8.8Hz , 2H), 8.26 (2, *J* = 8.8Hz , 2H), 8.83 (s, 1H);

^{13}C-NMR (100 MHz, $CDCl_3$, $CHCl_3$ = 77.0 ppm): δ = 27.9, 52.9, 54.8, 59.1, 79.8, 83.2, 120.1, 123.8, 128.3, 128.7, 129.6, 140.3, 141.1, 149.7, 164.1, 166.9, 168.0, 169.7.

HRMS (ESI): *m/z* calculated for $C_{24}H_{27}O_9N_3Na$ $[M + Na]^+$: 524.1645; found 524.1647.



To a stirred solution of (*2S,3R*)-1-*tert*-Butyl 4-methyl 2-methoxy-3-[4-(4-nitrobenzamido)benzamido]succinate (74.3 mg, 0.15 mmol) in 2.5 ml CH_2Cl_2 was added 1.5 ml TFA at room temperature. After stirring for 5h, the reaction mixture was added water and extracted with ethyl acetate. The combined organic phases were washed with water (three times), dried over Na_2SO_4 and concentrated under reduced pressure to give the title compound in quantitative yield (65.9 mg, quant.).

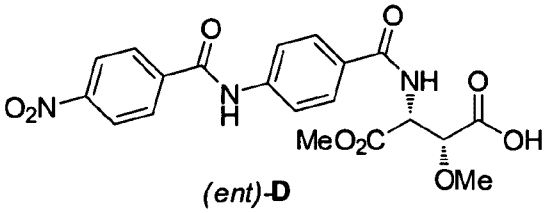
$[\alpha]_D^{20}$ = -16.4 ° (*c* 1.1, EtOAc);

^{1}H-NMR (400 MHz, DMSO, DMSO = 2.50 ppm): δ = 3.37 (s, 3H), 3.69 (s, *J* = 3H), 4.34 (d, *J* = 4.4Hz , 1H), 5.09 (dd, *J* = 4.8, 8.8Hz , 1H), 7.89-7.90 (m, 4H), 8.21 (dd, *J* = 2, 6.8Hz , 1H), 8.39 (dd, *J* = 2, 6.8Hz , 1H), 8.55 (d, *J* = 8.8Hz , 1H), 10.8 (s, 1H).

^{13}C-NMR (100 MHz, DMSO, DMSO = 40.0 ppm): δ = 52.9, 54.8, 58.7, 79.5, 120.0, 124.1, 129.0, 129.2, 129.8, 140.8, 142.2, 149.8, 164.7, 166.6, 170.2, 170.9.

HRMS (ESI): *m/z* calculated for $C_{20}H_{19}O_9N_3Na$ $[M + Na]^+$: 468.1019; found 468.1016.

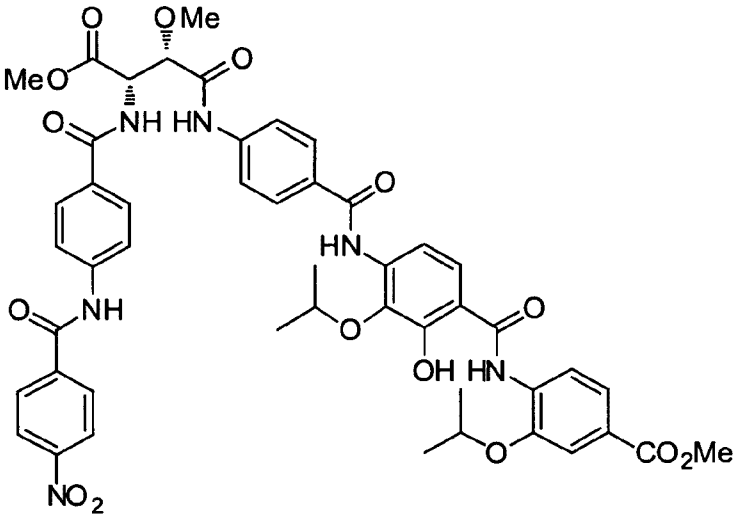
Optical rotation of other enantiomer:



(ent)-**D**

$[\alpha]_D^{20}$ = +13.9 ° (*c* 1.1, EtOAc);

Methyl-4-(4-(4-((2S,3S)-2,4-dimethoxy-3-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2- hydroxy-3-isopropxybenzamido)-3-isopropoxybenzoate



Methyl-4-[4-(4-aminobenzamido)-2-hydroxy-3-isopropxybenzamido]-3-isopropoxybenzoate (15.3 mg, 0.029 mmol) and (2*S*,3*R*)-2,4-dimethoxy-3-[4-(4-nitrobenzamido)benzamido]succinate (14.2 mg, 0.032 mmol) were dissolved in CH_2Cl_2 (3.4 mL) and cooled to 0 °C. Then, HOAt (5.9 mg, 0.044 mmol), DIPEA (7.7 µL, 0.044 mmol), and EDC·HCl (6.9 mg, 0.036 mmol) were added. The mixture was stirred from 0 °C to room temperature for 17 hours. The solvent was concentrated *in vacuo* to give an oily residue, which was purified by flash chromatography (petroleum ether /ethyl acetate = 94/6) to yield the title compound (20.1 mg, 0.021 mmol, 73%) as a colourless oil.

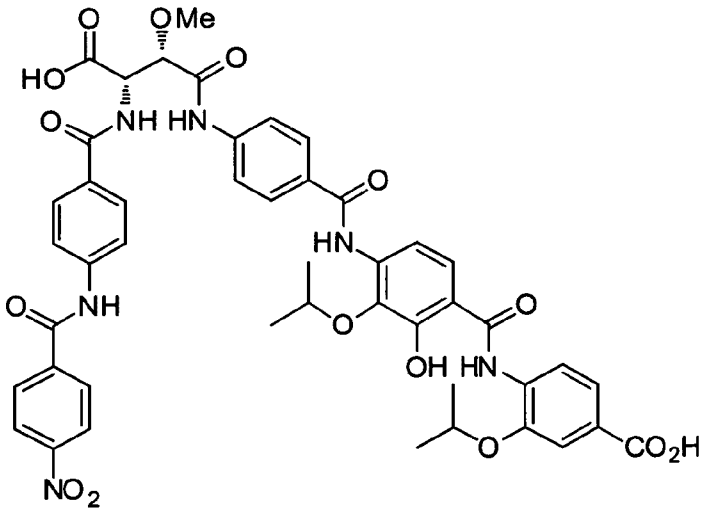
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H$_{-OH}$), 8.37 (d, *J* = 7.5 Hz, 2H), 8.20 (d, *J* = 7.5 Hz, 2H), 8.11 (s, 1H$_{-NH}$), 8.02 (s, 1H$_{-NH}$), 8.01 (d, *J* = 1.4 Hz, 2H), 7.98 (d, *J* = 7.5 Hz, 2H), 7.90 (d, *J* = 1.3 Hz, 1H), 7.81 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.78 (d, *J* = 7.4 Hz, 1H), 7.69 (d, *J* = 7.5 Hz, 1H), 7.61 (d, *J* = 7.5 Hz, 2H), 7.55 (s, 1H), 7.54 (s, 1H$_{-NH}$), 7.53 (s, 1H), 7.41 (d, *J* = 7.5 Hz, 1H), 5.72 (s, 1H$_{-NH}$), 5.63 (s, 1H$_{-NH}$), 5.10 (d, *J* = 3.8 Hz, 1H), 4.76 (d, *J* = 3.8 Hz, 1H), 4.04 – 3.98 (m, 2H), 3.97 (s, *J* = 3.1 Hz, 3H), 3.74 (s, 3H), 3.32 (s, 3H), 1.47 (d, *J* = 5.7 Hz, 6H), 1.39 (d, *J* = 5.7 Hz, 6H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.30, 168.15, 168.07, 167.77, 166.93, 166.88, 166.82, 158.83, 151.01, 146.97, 140.78, 139.42, 138.71, 134.97, 134.55, 131.57, 130.00, 130.00, 129.41, 129.41, 129.39, 129.39, 128.12, 127.53, 127.24, 124.17, 124.17, 123.28, 122.61, 122.61, 121.78, 121.78, 121.44, 115.94, 114.88, 113.30,

106.09, 78.00, 75.89, 74.13, 58.51, 56.50, 52.17, 52.08, 21.80, 21.80, 21.80, 21.80 ppm.

HRMS (ESI): Calculated for $C_{48}H_{47}N_6O_{15}$ $(M-H)^-$: 947.3178, found: 947.3175.

Cystobactamide A



Methyl-4-4-[4-((2*S*,3*S*)-2,4-dimethoxy-3-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido]benzamido}-2-hydroxy-3-isopropxybenzamido)-3-isopropoxybenzoate (15.2 mg, 0.016 mmol) was dissolved in a mixture 1/1 of THF/H_2O (0.2/0.2 mL). Then, solid LiOH (3.8 mg, 0.16 mmol) was added and the reaction mixture was stirred at room temperature for 17 hours. The aqueous layer was acidified with 1M HCl until pH~1 and extracted with ethyl acetate (3x). The organic extracts were combined, dried over $MgSO_4$ and filtered. The solvent was concentrated *in vacuo* to yield the title compound (13.3 mg, 0.014 mmol, 90%) as a yellow wax.

$[\alpha]_D^{20}$ = -19.1 ° (*c* 1.1, EtOAc)

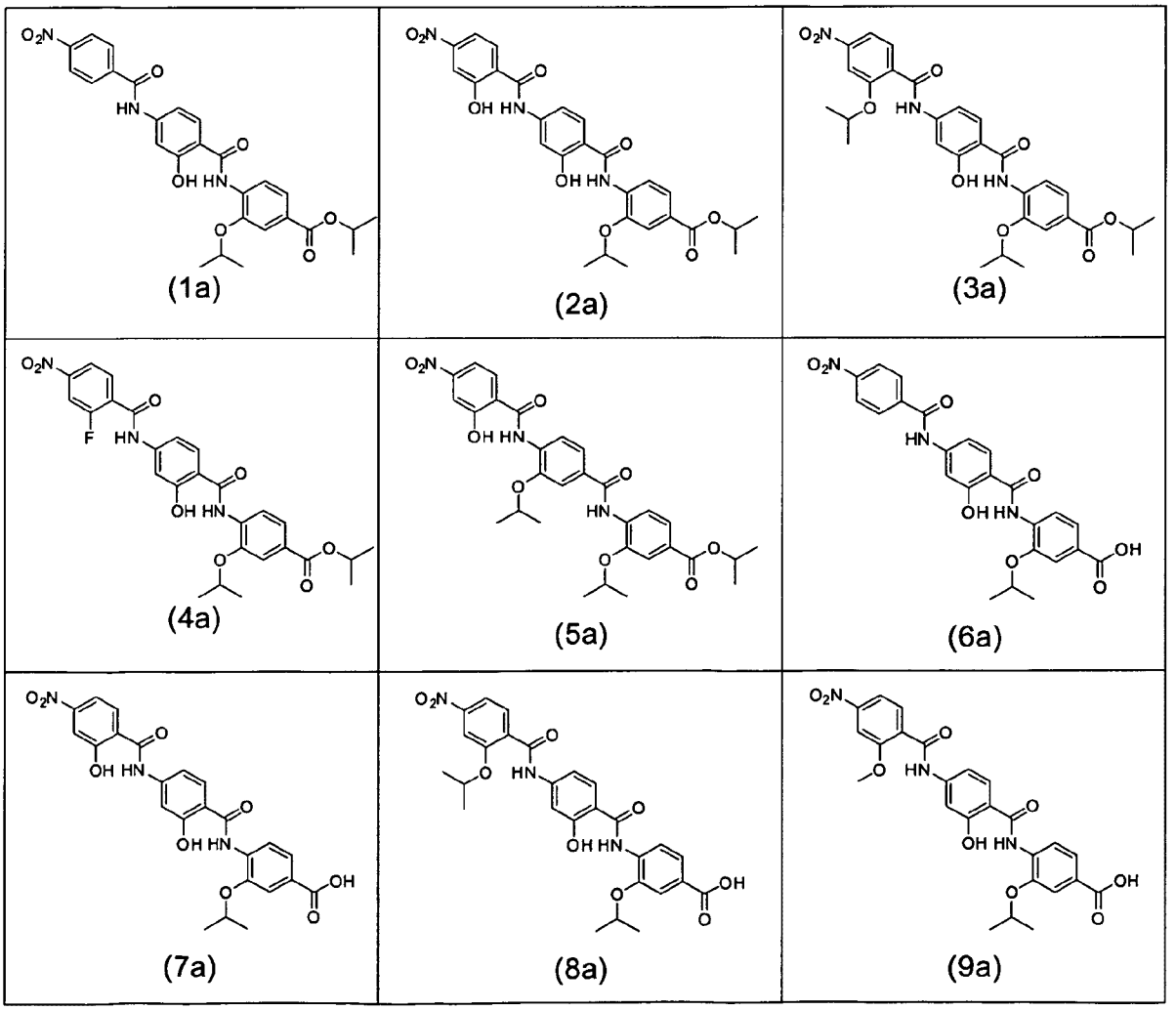
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 7.5 Hz, 2H), 8.15 (d, *J* = 7.5 Hz, 2H), 8.00 (d, *J* = 1.8 Hz, 2H), 7.98 (d, *J* = 1.8 Hz, 2H), 7.90 (d, *J* = 1.8 Hz, 1H), 7.86 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.78 (d, *J* = 7.5 Hz, 1H), 7.65 (s, 1H), 7.63 (d, *J* = 7.5 Hz, 2H), 7.58 (s, $1H_{-NH}$), 7.54 (d, *J* = 7.5 Hz, 2H), 7.51 (s, $1H_{-NH}$), 7.10 (s, $1H_{-NH}$), 7.03 (d, *J* = 7.5 Hz, 1H), 6.35 (s, $1H_{-NH}$), 5.57 (s, $1H_{-NH}$), 5.42 (s, $1H_{-OH}$), 4.93 (s, 1H), 4.70 (s, 1H),

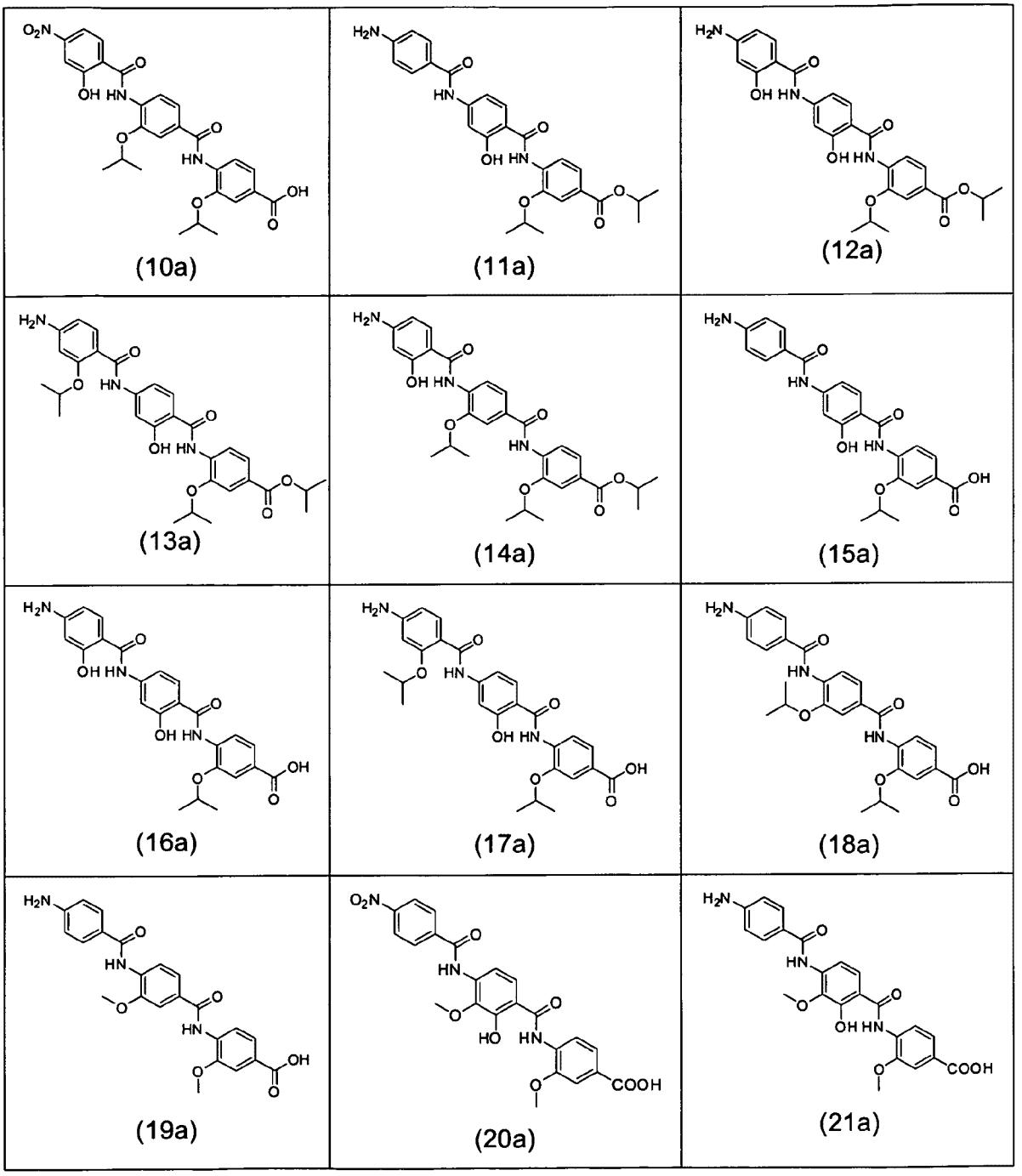
4.01 (hept, J = 5.6 Hz, 1H), 3.95 (hept, J = 5.6 Hz, 1H), 3.38 (s, 3H), 1.48 (s, 6H), 1.47 (s, 6H) ppm.

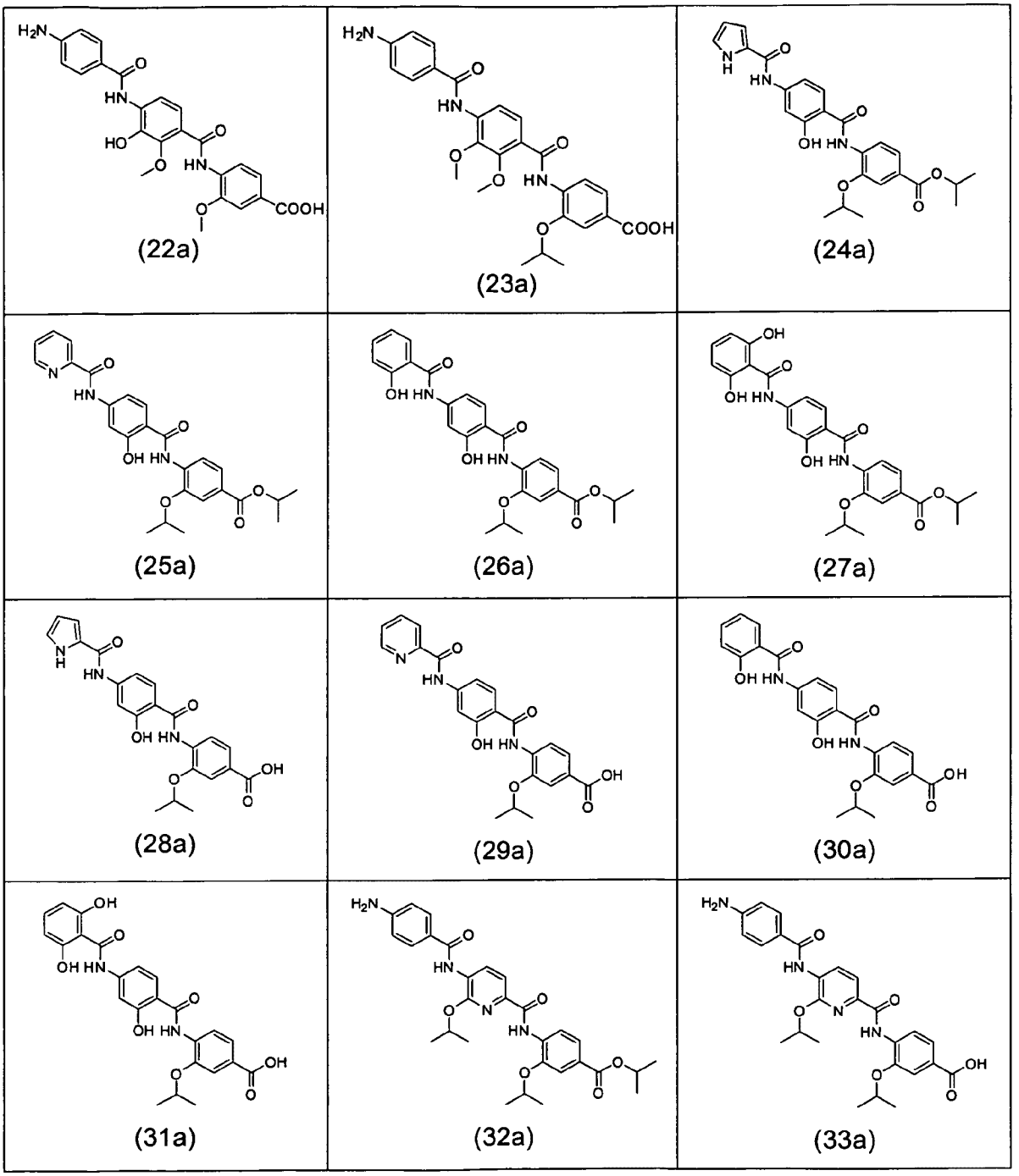
^{13}C NMR (100 MHz $CDCl_3$) δ 173.30, 169.54, 168.18, 168.07, 167.77, 166.88, 166.82, 158.83, 151.01, 149.88, 140.78, 139.42, 138.71, 136.26, 134.97, 134.55, 130.00, 130.00, 129.41, 129.41, 129.39, 129.39, 128.12, 127.53, 125.15, 124.17, 124.17, 123.28, 122.84, 122.61, 122.61, 121.78, 121.78, 120.41, 113.82, 113.30, 106.09, 77.86, 75.89, 74.13, 58.51, 54.58, 21.80, 21.80, 21.80, 21.80 ppm.

HRMS (ESI): Calculated for $C_{46}H_{43}N_6O_{15}$ $(M-H)^-$: 920.2865, found: 920.2866.

Synthesis of Cystobactamide C derivatives



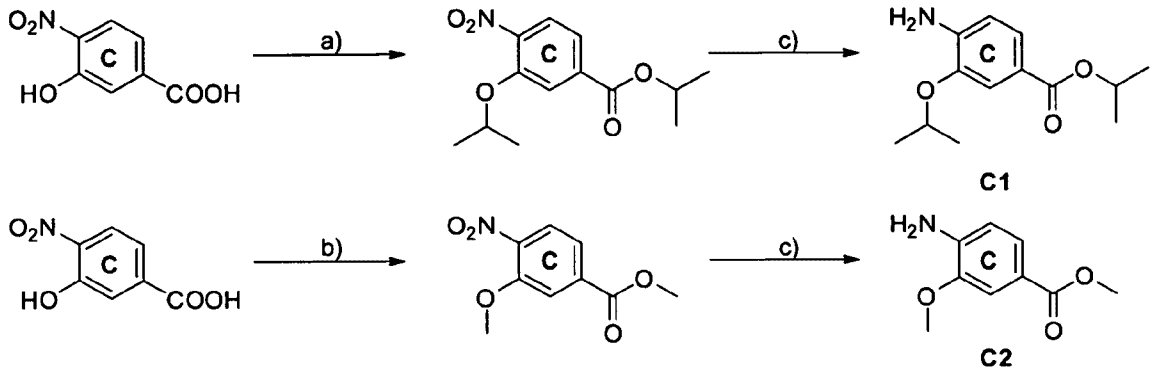

(10a)
(11a)
(12a)
(13a)
(14a)
(15a)
(16a)
(17a)
(18a)
(19a)
(20a)
(21a)



1.1. Synthesis of the different used individual rings

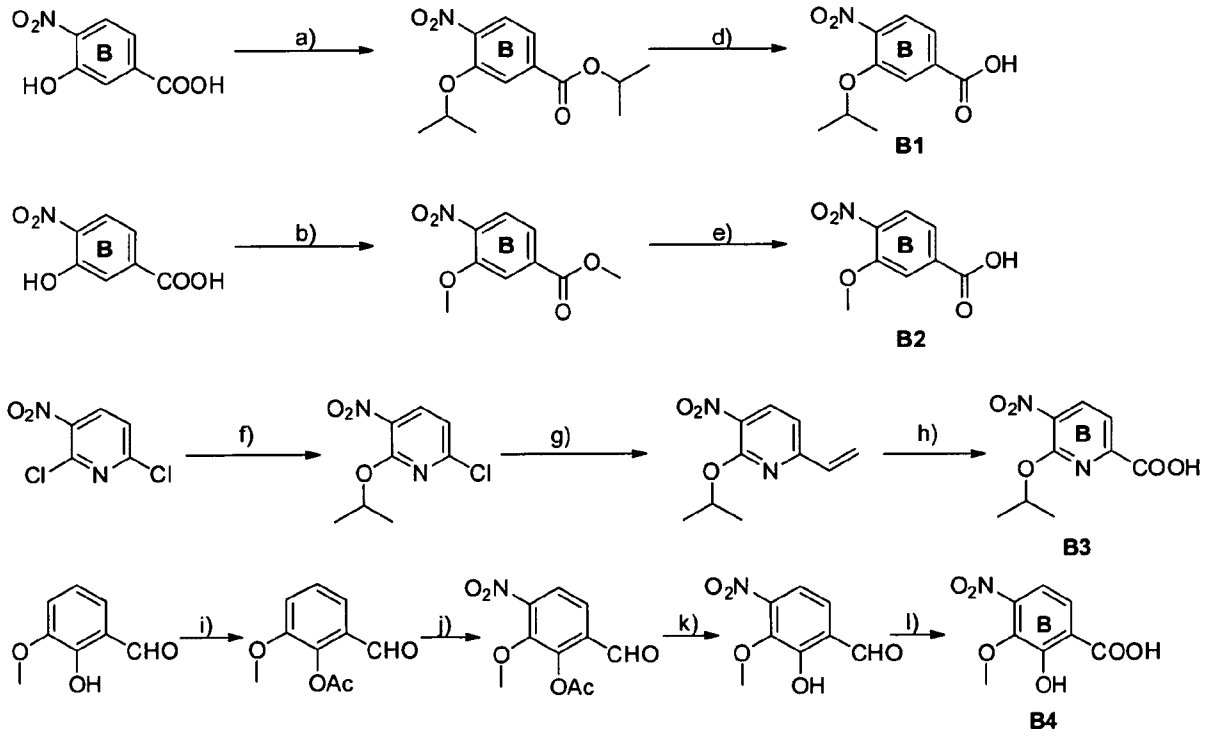
The preparation of the different individual rings that were used during the synthesis of the cystobactamide C derivatives is described here.

Preparation of Ring C



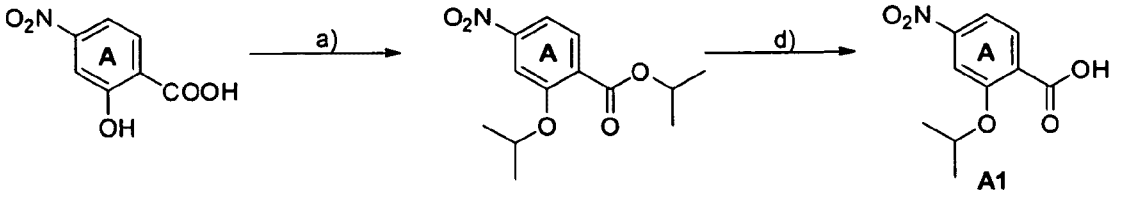
a) $BrCH(CH_3)_2$, K_2CO_3, DMF, 90°C, overnight; b) $SO_2(OMe)_2$, K_2CO_3, DMF, 90°C, overnight; c) Fe, NH_4Cl, $EtOH/H_2O$, reflux, 2 hours

Preparation of Ring B



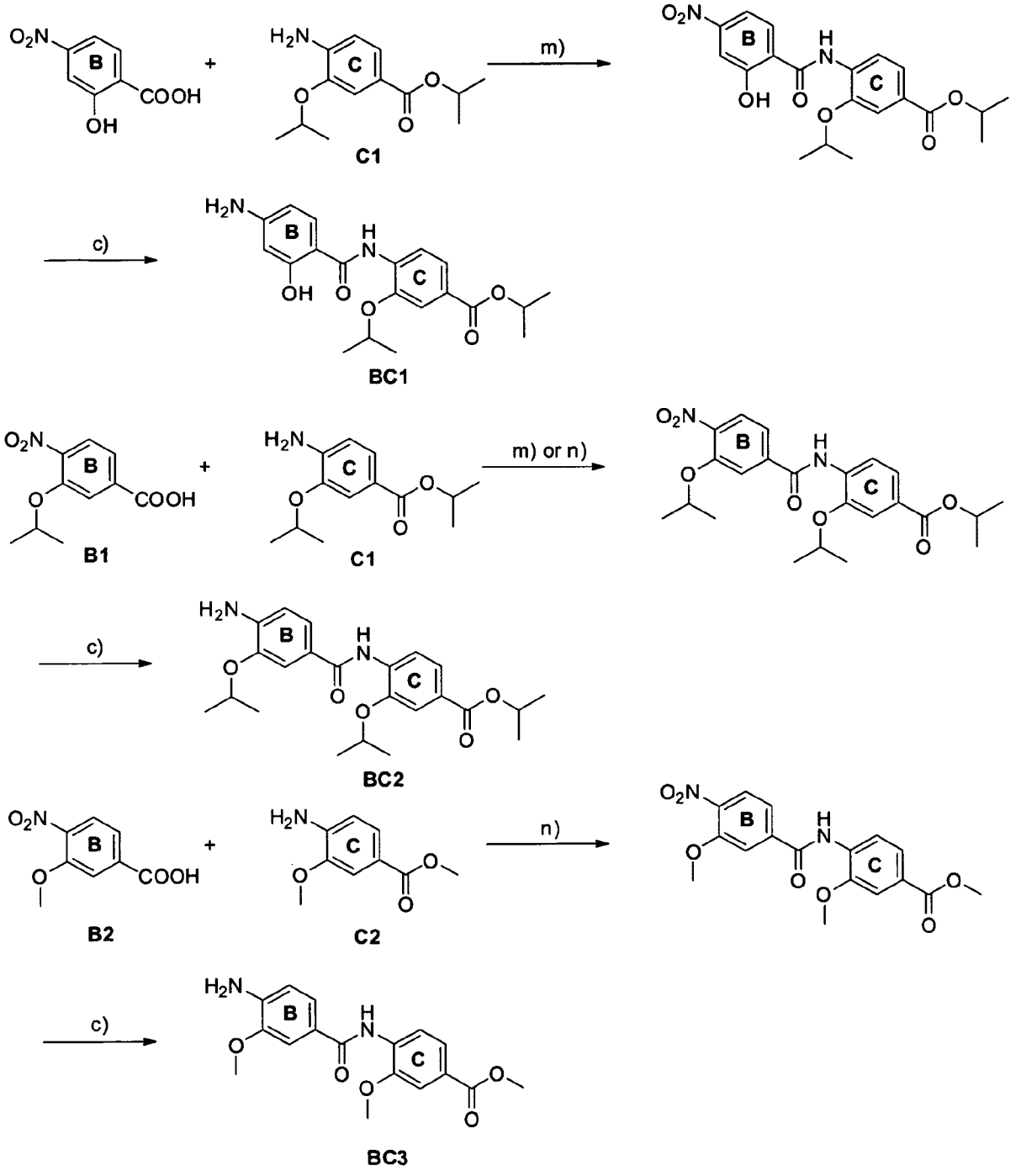
a) $BrCH(CH_3)_2$, K_2CO_3, DMF, 90°C, overnight; b) $SO_2(OMe)_2$, K_2CO_3, DMF, 90°C, overnight; d) NaOH/MeOH, 45°C, overnight; e) KOH, $MeOH/H_2O$; f) *i*-PrOH/NaH; g) $H_2C{=}CHSn(Bu)_3$, $Pd[(Ph)_3P]_4$; h) $KMnO_4$; i) AcCl/pyridine; j) KNO_3/TFAA, k) NaOH, l) $AgNO_3$/NaOH

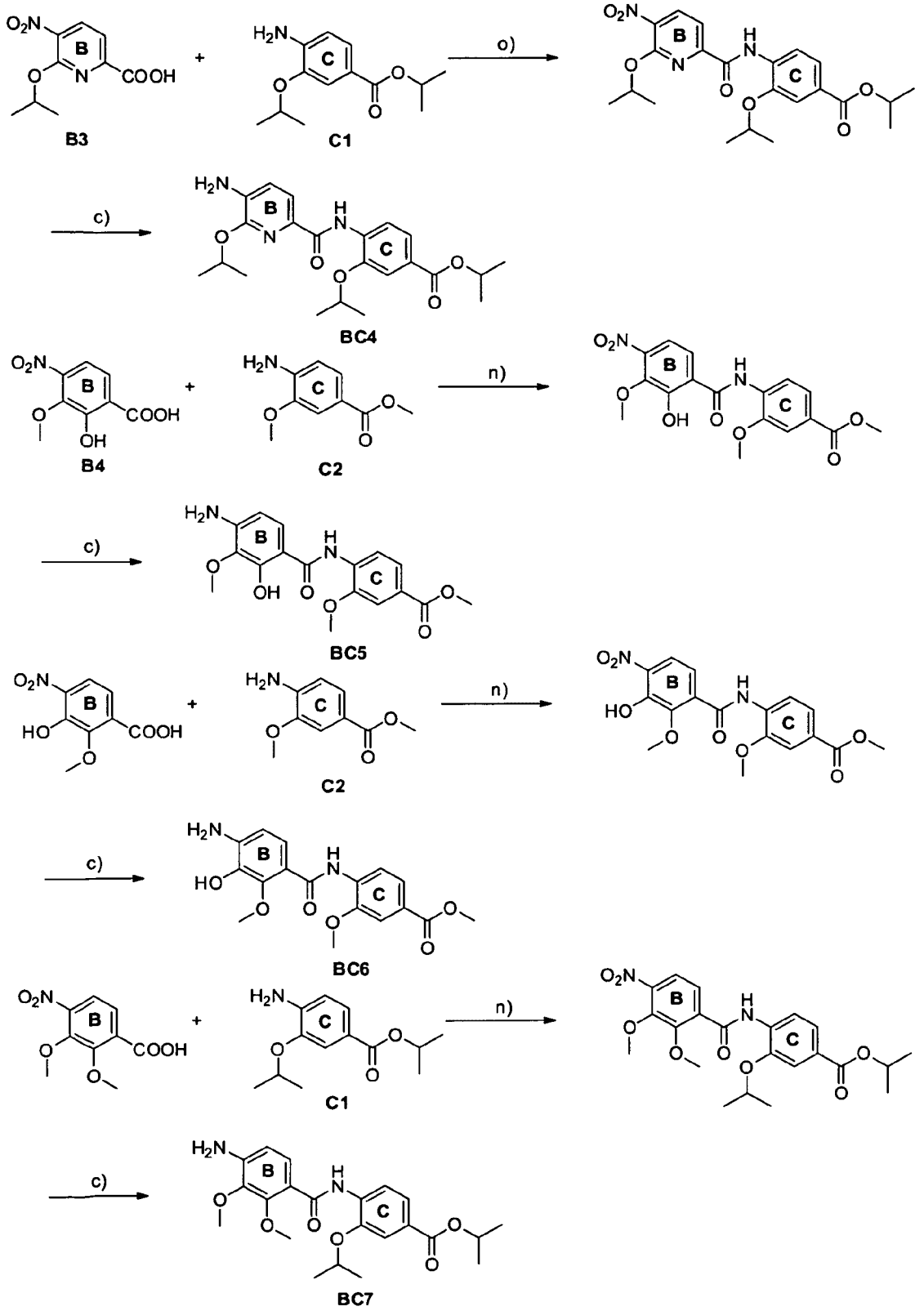
Preparation of Ring A



a) $BrCH(CH_3)_2$, K_2CO_3, DMF, 90°C, overnight; d) NaOH/MeOH, 45°C, overnight

1.2. Coupling of Ring B and C to give the different prepared BC fragments

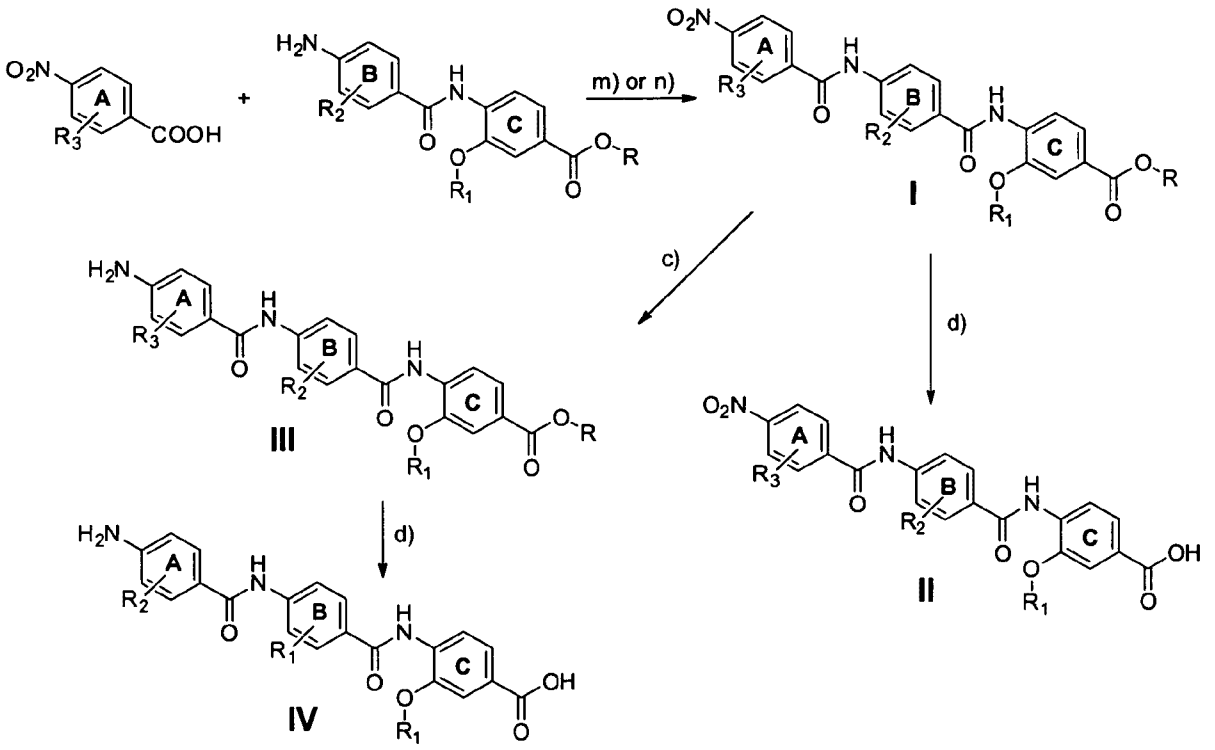

m)
C1
c)
BC1
B1
m) or n)
BC2
B2
n)
BC3



c) Fe, NH_4Cl, EtOH/H_2O, reflux, 2 hours; m) PCl_3, CH_2Cl_2, Xylene, 145°C, 2 hours; n) Cl_2PPh_3, $CHCl_3$; o) EDC, HOBT

1.3. Coupling of Ring A with BC fragments

1.3.1. Coupling of Ring A with BC fragments (BC1, BC2, BC3, BC5, BC6, BC7) to synthesize the Cystobactamide C derivatives (1a) - (23a)

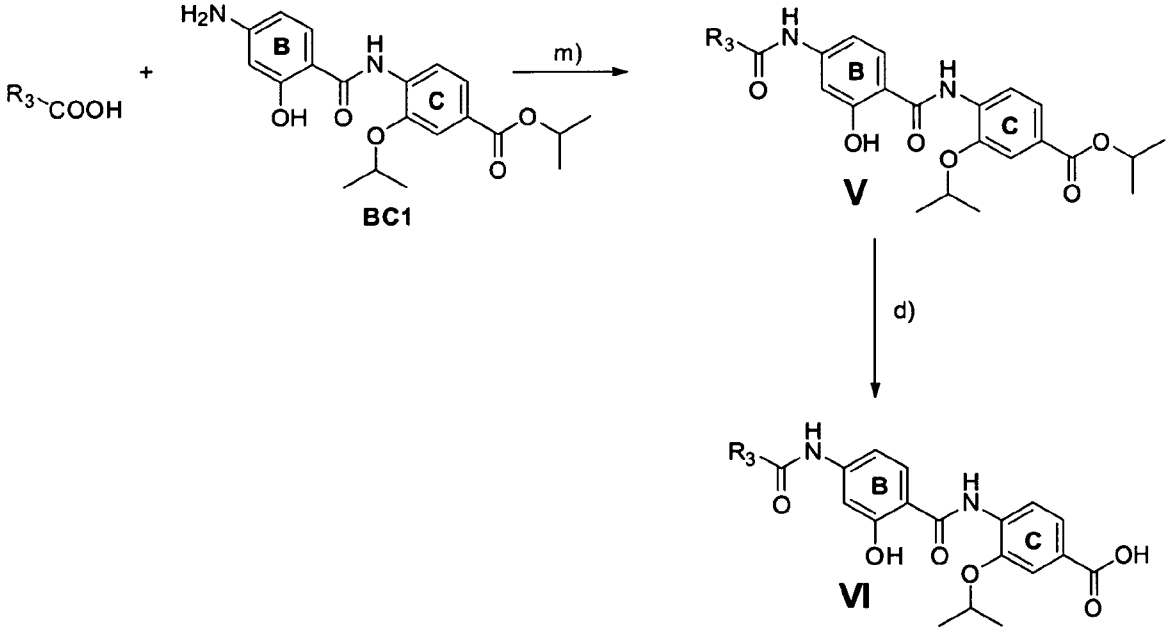


c) Fe, NH_4Cl, $EtOH/H_2O$, reflux, 2 hours; d) NaOH/MeOH, 45°C, overnight; m) PCl_3, CH_2Cl_2, Xylene, 145°C, 2 hours; n) Cl_2PPh_3, $CHCl_3$

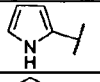
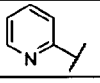
Compound	Scaffold	R	R_1	R_2	R_3
(1a)	I	*i*Pr	*i*Pr	2-OH	H
(2a)	I	*i*Pr	*i*Pr	2-OH	2-OH
(3a)	I	*i*Pr	*i*Pr	2-OH	2-O*i*Pr
(4a)	I	*i*Pr	*i*Pr	2-OH	2-F
(5a)	I	*i*Pr	*i*Pr	3-O*i*Pr	2-OH
(6a)	II	-	*i*Pr	2-OH	H
(7a)	II	-	*i*Pr	2-OH	2-OH
(8a)	II	-	*i*Pr	2-OH	2-O*i*Pr
(9a)	II	-	*i*Pr	2-OH	2-OMe
(10a)	II	-	*i*Pr	3-O*i*Pr	2-OH
(11a)	III	*i*Pr	*i*Pr	2-OH	H

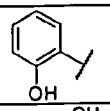
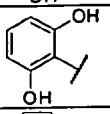
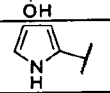
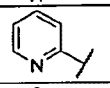
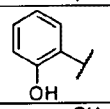
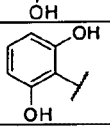
(12a)	III	*i*Pr	*i*Pr	2-OH	2-OH
(13a)	III	*i*Pr	*i*Pr	2-OH	2-O*i*Pr
(14a)	III	*i*Pr	*i*Pr	3-O*i*Pr	2-OH
(15a)	IV	-	*i*Pr	2-OH	H
(16a)	IV	-	*i*Pr	2-OH	2-OH
(17a)	IV	-	*i*Pr	2-OH	2-O*i*Pr
(18a)	IV	-	*i*Pr	3-O*i*Pr	H
(19a)	IV	-	Me	3-OMe	H
(20a)	II	-	Me	2-OH, 3OMe	H
(21a)	IV	-	Me	2-OH, 3OMe	H
(22a)	IV	-	Me	2-OMe, 3OH	H
(23a)	IV	-	*i*Pr	2,3-diOMe	H

1.3.2. Coupling of Ring A with BC1 fragment to synthesize the Cystobactamide C derivatives (24a) - (31a)

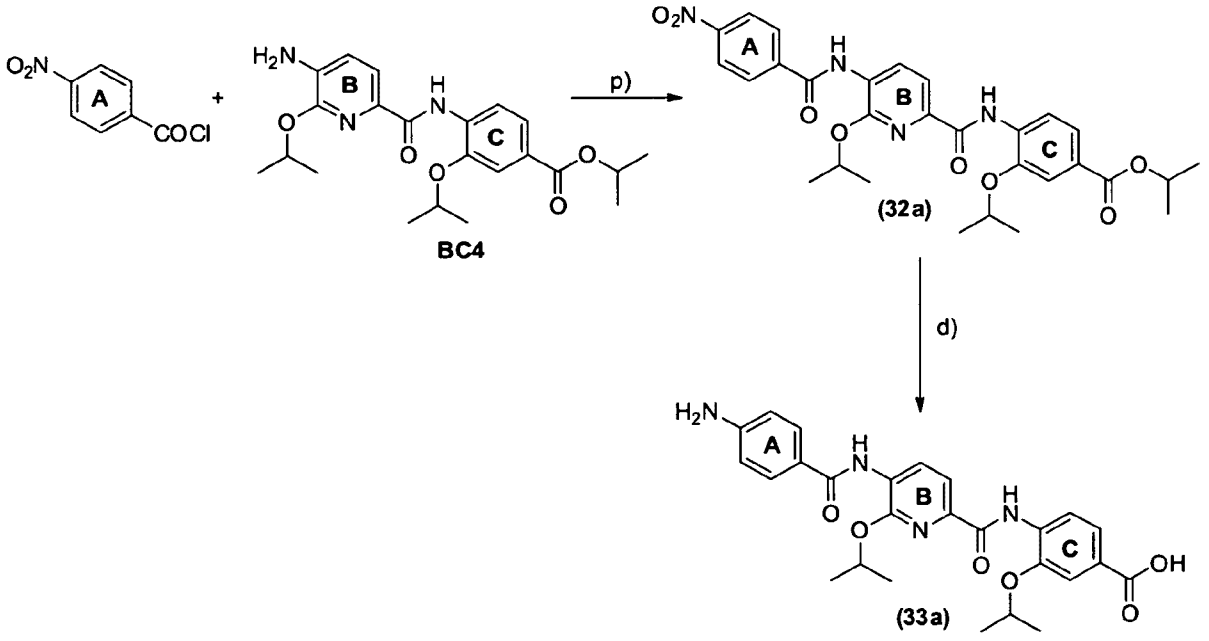


d) NaOH/MeOH, 45°C, overnight; m) PCl_3, CH_2Cl_2, Xylene, 145°C, 2 hours

Compound	Scaffold	R_3
(24a)	V	
(25a)	V	

(26a)	V	
(27a)	V	
(28a)	VI	
(29a)	VI	
(30a)	VI	
(31a)	VI	

1.3.3. Coupling of Ring A with BC4 fragment to synthesize the Cystobactamide C derivatives (32a) - (33a)



d) NaOH/MeOH, 45°C, overnight; p) CH_2Cl_2, pyridine, rt, overnight

2. Experimental

2.1. General Experimental information

Starting materials and solvents were purchased from commercial suppliers, and used without further purification. All chemical yields refer to purified compounds, and not optimized. Reaction progress was monitored using TLC Silica gel 60 F_{254} aluminium sheets, and visualization was accomplished by UV at 254 nm. Flash chromatography was performed using silica gel 60 Å (40-63 µm). Preparative RP-HPLC was carried out on a Waters Corporation setup contains a 2767 sample manager, a 2545 binary gradient module, a 2998 PDA detector and a 3100 electron spray mass spectrometer. Purification was performed using a Waters XBridge column (C18, 150 × 19 mm, 5 µm), a binary solvent system A and B (A = water with 0.1% formic acid; B = MeCN with 0.1% formic acid) as eluent, a flow rate of 20 mL/min and a gradient of 60% to 95% B in 8 min were applied. Melting points were determined on a Stuart Scientific melting point apparatus SMP3 (Bibby Sterilin, UK), and are uncorrected. NMR spectra were recorded either on Bruker DRX-500 (^{1}H, 500 MHz; ^{13}C, 126 MHz), or Bruker Fourier 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at 300 K. Chemical shifts are recorded as δ values in ppm units by reference to the hydrogenated residues of deuterated solvent as internal standard ($CDCl_3$: δ = 7.26, 77.02; DMSO-d_6: δ = 2.50, 39.99). Splitting patterns describe apparent multiplicities and are designated as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet). Coupling constants (*J*) are given in Hertz (Hz). Purity of all compounds used in biological assays was ≥95% as measured by LC/MS Finnigan Surveyor MSQ Plus (Thermo Fisher Scientific, Dreieich, Germany). The system consists of LC pump, autosampler, PDA detector, and single-quadrupole MS detector, as well as the standard software Xcalibur for operation. RP C18 Nucleodur 100-5 (125 × 3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase, and a binary solvent system A and B (A = water with 0.1% TFA; B = MeCN with 0.1% TFA) was used as mobile phase. In a gradient run the percentage of B was increased from an initial concentration of 0% at 0 min to 100% at 15 min and kept at 100% for 5 min. The injection volume was 10 µL and flow rate was set to 800 µL/min. MS (ESI) analysis was carried out at a spray voltage of 3800 V, a capillary temperature of 350 °C and a source CID of 10 V. Spectra were acquired in positive mode from 100 to 1000 m/z and at 254 nm for UV tracing.

2.2. LC/MS data for the triaryl derivatives

Compound	LC/MS *m/z* (ESI+)
(1a)	521.99 $[M + H]^+$
(2a)	537.87 $[M + H]^+$
(3a)	579.90 $[M + H]^+$
(4a)	540.07 $[M + H]^+$
(5a)	580.11$[M + H]^+$
(6a)	479.98 $[M + H]^+$
(7a)	496.02 $[M + H]^+$
(8a)	537.99 $[M + H]^+$
(9a)	509.98 $[M + H]^+$
(10a)	538.11 $[M + H]^+$
(11a)	492.02 $[M + H]^+$
(12a)	508.01 $[M + H]^+$
(13a)	550.02 $[M + H]^+$
(14a)	550.13 $[M + H]^+$
(15a)	449.87 $[M + H]^+$
(16a)	465.93 $[M + H]^+$
(17a)	508.07 $[M + H]^+$
(18a)	492 $[M + H]^+$
(19a)	435 $[M]^+$
(20a)	482 $[M + H]^+$
(21a)	452 $[M + H]^+$
(22a)	452 $[M + H]^+$
(23a)	494 $[M + H]^+$
(24a)	466.20 $[M + H]^+$
(25a)	478.07 $[M + H]^+$
(26a)	493.17 $[M + H]^+$
(27a)	509.12 $[M + H]^+$
(28a)	423.53 $[M + H]^+$

(29a)	436.13 $[M + H]^+$
(30a)	451.10 $[M + H]^+$
(31a)	467.11 $[M + H]^+$
(32a)	535 $[M + H]^+$
(33a)	493 $[M + H]^+$

2.3 General synthetic procedures:

a) A mixture of the acid (25 mmol), isopropyl bromide (52 mmol) and potassium carbonate (52 mmol) in 100ml DMF were heated overnight at 90°C. Excess DMF was then removed under reduced pressure and the remaining residue was partitioned between water and ethyl acetate. The organic layer was dried over sodium sulphate and the excess solvent was then removed under reduced pressure to give the pure product.

c) To a stirred solution of the nitro derivative (10 mmol) in EtOH (60 mL), iron powder (2.80 g, 50 mmol) was added at 55 °C followed by NH_4Cl (266 mg, 5 mmol) solution in water (30 mL). The reaction was refluxed for 1-2 h, then iron was filtered while hot and the filtrate was concentrated under vacuum till dryness. The residue was diluted with water (30 mL) and basified by $NaHCO_3$ (saturated aqueous solution) to pH 7–8. The mixture was extracted with EtOAc. The combined organic extract was washed with brine, dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The obtained crude material was triturated with n-hexane, and collected by filtration.

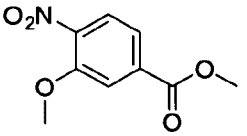
d) Ester hydrolysis was done according to the following reported procedure.[1] The ester (0.1 mmol), sodium hydroxide 1M (3 mL) and anhydrous methanol were heated overnight at 45°C. On cooling, the reaction mixture was acidified to pH 1 (3 mL, hydrochloric acid 1 M) and extracted with dichloromethane (3 x 150 mL). The organic was dried over sodium sulphate and the solvent removed under reduced pressure to leave give the pure product.

m) Amide formation was done according to the following reported procedure.[2] A boiling solution of the acid (1 mmol) and the amine (1mmol) in xylenes 2.5 ml was treated with a 2M solution of PCl_3 in CH_2Cl_2 (0.4 mmol). After 2 hours the excess solvent was evaporated and the residue was purified using column chromatography.

n) To a stirred solution of the acid (2 mmol), amine (2.4 mmol) in anhydrous $CHCl_3$ (50 mL) under a nitrogen atmosphere, dichlorotriphenylphosphorane (3.0 g, 9 mmol) was added. The reaction was heated at 80 °C for 5 h. Solvent was removed by vacuum distillation. The residue was then purified using flash chromatography.

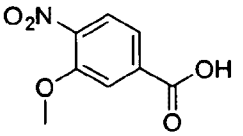
2.4 Specific synthetic procedures:

Methyl 3-methoxy-4-nitrobenzoate



To a stirred mixture of 3-hydroxy-4-nitrobenzoic acid (9.16 g, 50 mmol) and K_2CO_3 (15.2 g, 110 mmol) in DMF (150 mL), dimethyl sulfate (25.2 g, 200 mmol) was added portion wise then the reaction was stirred at 90 °C overnight. After cooling the mixture was poured on to ice cooled water (400 mL), the precipitate was filtered, washed with cold water then *n*-hexane.
Yield 95% (pale yellow solid), m/z (ESI+) 212 $[M + H]^+$.

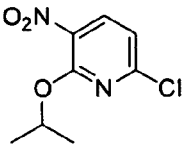
3-Methoxy-4-nitrobenzoic acid



To a stirred solution of methyl 3-methoxy-4-nitrobenzoate (2.11 g, 10 mmol) in MeOH (30 mL), KOH (1.68 g, 30 mmol) in water (30 mL) was added. The reaction was refluxed for 2 h then MeOH was evaporated by vacuum distillation. The residue was diluted with water (20 mL). The solution was cooled in an ice bath and acidified by

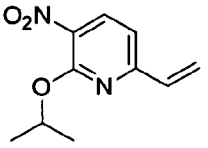
$KHSO_4$ (saturated aqueous solution) to pH 3–4. The precipitated solid was collected by filtration, washed with cold water then *n*-hexane.
Yield 96% (off-white solid), m/z (ESI+) 198 $[M + H]^+$.

6-Chloro-2-isopropoxy-3-nitropyridine



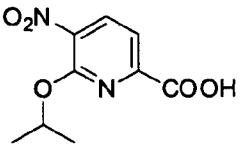
To a stirred solution of 2,6-dichloro-3-nitropyridine (3.86 g, 20 mmol) in toluene (30 mL), isopropanol (1.44 g, 24 mmol) was added. The mixture was stirred at 0 °C for 15 min. then NaH (50–60% in mineral oil, 1.22 g, 28 mmol) was added portion wise under a nitrogen atmosphere, and the reaction was allowed to stir at room temperature overnight. The reaction was quenched with brine, then diluted with water and extracted with EtOAc. The combined organic extract was washed with brine, dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The residue was dissolved in toluene and purified using flash chromatography (SiO_2, *n*-hexane–EtOAc = 5:1).
Yield 70% (yellowish white crystals), m/z (ESI+) 217 $[M + H]^+$.

2-Isopropoxy-3-nitro-6-vinylpyridine



To a stirred solution of 6-chloro-2-isopropoxy-3-nitropyridine (650 mg, 3 mmol), and tributyl(vinyl)tin (1.0 g, 3.15 mmol) in toluene (20 mL) under a nitrogen atmosphere, tetrakis(triphenylphosphine) palladium(0) (180 mg, 5% eq.) was added. The reaction was refluxed overnight. Brine was added, and the reaction was extracted with EtOAc. The combined organic extract was washed with brine, dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The crude product was used directly in the next step without further purification. Yield 90% (yellow liquid), m/z (ESI+) 208 $[M]^+$.

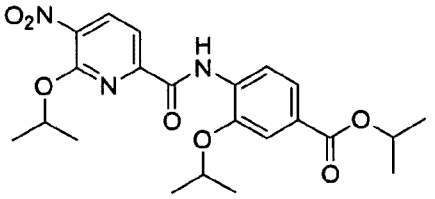
6-Isopropoxy-5-nitropyridine-2-carboxylic acid



To a stirred solution of 2-isopropoxy-3-nitro-6-vinylpyridine (625 mg, 3 mmol) in acetone (10 mL), $KMnO_4$ (1.9 g, 12 mmol) solution in 50% aq. acetone (50 mL) was added. The reaction was stirred at room temperature for 24 h. NaOH 0.5 M (5 mL) was added, then the mixture was filtered and filtrate was concentrated under vacuum. The residue was cooled in an ice bath and carefully acidified by $KHSO_4$ (saturated aqueous solution) to pH 4–5, then extracted with EtOAc. The combined organic extract was washed with brine, dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The obtained crude material was triturated with n-hexane, and collected by filtration.

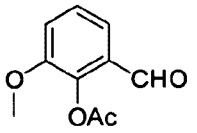
Yield 75% (beige solid), m/z (ESI+) 227 $[M + H]^+$.

Isopropyl 3-isopropoxy-4-{[(6-isopropoxy-5-nitropyridin-2-yl)carbonyl]amino}benzoate



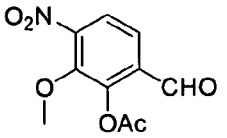
To a stirred solution of 6-isopropoxy-5-nitropyridine-2-carboxylic acid (226 mg, 1 mmol), and isopropyl 4-amino-3-isopropoxybenzoate (237 mg, 1 mmol) in a mixture of anhydrous $CHCl_3$ (5o mL) and DMF (1 mL) under a nitrogen atmosphere, HOBt (676 mg, 5 mmol) was added at 0 °C followed by EDC.HCl (958 mg, 5 mmol). The reaction was allowed to stir at 0 °C for 2 h. then at room temperature overnight. Solvent was removed by vacuum distillation. The residue was dissolved in toluene and purified using flash chromatography (SiO_2, *n*-hexane–EtOAc = 2:1). Yield 70% (pale yellow solid), m/z (ESI+) 446 $[M + H]^+$.

2-formyl-6-methoxyphenyl acetate



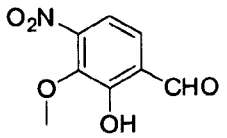
To a stirred solution of 3-methoxysalicylaldehyde (4.56 g, 30 mmol), and pyridine (2.43 mL, 30 mmol) in DCM (40 mL), acetyl chloride (2.36 g, 30 mmol) was added drop wise. The reaction was stirred at room temperature overnight then the solvent was removed by vacuum distillation. The residue was triturated in cold dil. HCl and filtered, washed with cold water then *n*-hexane.
Yield 94% (off-white solid), m/z (ESI+) 195 $[M + H]^+$.

6-formyl-2-methoxy-3-nitrophenyl acetate



To a stirred ice-cooled suspension of 2-formyl-6-methoxyphenyl acetate (1.94 g, 10 mmol), and KNO_3 (1.01 g, 10 mmol) in $CHCl_3$ (15 mL), trifluoroacetic anhydride (12 mL) was added. The reaction was stirred in an ice bath for 2 h. then at room temperature overnight. The reaction was diluted very carefully with water (50 mL) and extracted with $CHCl_3$. The combined organic extract was dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The residue was dissolved in toluene and purified using flash chromatography (SiO_2, *n*-hexane–EtOAc = 3:1). Yield 45% (yellow semisolid), m/z (ESI+) 239 $[M]^+$.

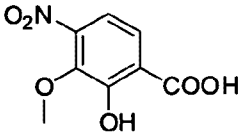
2-hydroxy-3-methoxy-4-nitrobenzaldehyde



To a stirred suspension of 6-formyl-2-methoxy-3-nitrophenyl acetate (957 mg, 4 mmol) in water (30 mL), NaOH (0.8 g, 20 mmol) was added. The reaction was refluxed for 2 h then allowed to stir at room temperature overnight. The solution was cooled in an ice bath and acidified by HCl 2 M to pH 3–4. The precipitated solid was collected by filtration, washed with cold water then *n*-hexane.

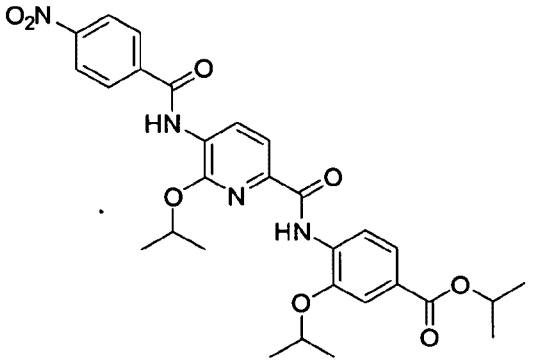
Yield 90% (yellowish brown solid), m/z (ESI+) 197 $[M]^+$.

2-hydroxy-3-methoxy-4-nitrobenzoic acid



To a stirred solution of 2-hydroxy-3-methoxy-4-nitrobenzaldehyde (788 mg, 4 mmol), and NaOH (0.8 g, 20 mmol) in water (50 mL), $AgNO_3$ (3.4 g, 20 mmol) was added portion wise. The reaction was refluxed overnight, then allowed to cool and filtered through celite. Filtrate was cooled in an ice bath and acidified with HCl 37% to pH 3–4. The precipitated solid was collected by filtration, washed with cold water then *n*-hexane. Yield 65% (beige solid), m/z (ESI+) 213 $[M]^+$.

Isopropyl 3-isopropoxy-4-[({6-isopropoxy-5-[(4-nitrobenzoyl)amino]pyridin-2-yl}carbonyl)amino]benzoate



To a stirred solution of isopropyl 4-{[(5-amino-6-isopropoxypyridin-2-yl)carbonyl]amino}-3-isopropoxybenzoate (207 mg, 0.5 mmol), and pyridine (0.1 mL) in DCM (20 mL), 4-nitrobenzoyl chloride (185 mg, 1 mmol) was added. The reaction was stirred at room temperature overnight then the HCl 2 M (20 mL) was added. The mixture was extracted with DCM then EtOAc. The combined organic extract was dried ($MgSO_4$), and the solvent was removed by vacuum distillation. The residue was dissolved in toluene and purified using flash chromatography (SiO_2, *n*-hexane–EtOAc = 1:1). Yield 80% (yellow crystals), m/z (ESI+) 565 $[M + H]^+$.

5. References:

1) Valeria Azzarito, Panchami Prabhakaran, Alice I. Bartlett, Natasha Murphy, Michaele J. Hardie, Colin A. Kilner, Thomas A. Edwards, Stuart L. Warriner, Andrew J. Wilson. *2-O-Alkylated Para-Benzamide α-Helix Mimetics: The Role of Scaffold Curvature*. Org. Biomol. Chem., 2012, 10, 6469.

2) Alina Fomovska, Richard D. Wood, Ernest Mui, Jitenter P. Dubey, Leandra R. Ferreira, Mark R. Hickman, Patricia J. Lee, Susan E. Leed, Jennifer M. Auschwitz, William J. Welsh, Caroline Sommerville, Stuart Woods, Craig Roberts, and Rima McLeod. *Salicylanilide Inhibitors of Toxoplasma gondii.* J. Med. Chem., 2012, 55 (19), pp 8375–8391.

6. Activity of these compounds

Several of these compounds were tested for their activity against an *E. coli* strain (TolC-deficient) according to the procedures described above. Most tested compounds showed an activity (MIC) of from 1 to 320 μM.

CLAIMS:

1. A compound of formula (I)

$$R^1\text{-}Ar^1\text{-}L^1\text{-}Ar^2\text{-}L^2\text{-}Ar^3\text{-}L^3\text{-}Ar^4\text{-}L^4\text{-}Ar^5\text{-}R^2$$

(I)

wherein

Ar^1 is an optionally substituted 1,4-phenylene group;

Ar^2 is an optionally substituted 1,4-phenylene group;

Ar^3 is an optionally substituted 1,4-phenylene group;

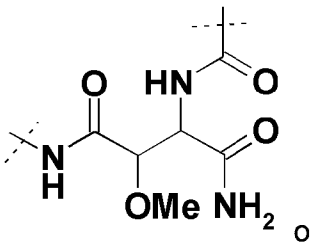
Ar^4 is an optionally substituted 1,4-phenylene group;

Ar^5 is an optionally substituted 1,4-phenylene group;

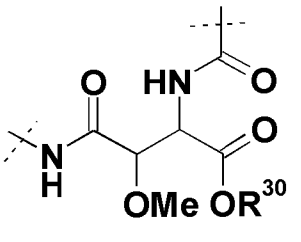
L^1 is NHCO (wherein the nitrogen atom is bound to Ar^1);

L^2 is NHCO (wherein the nitrogen atom is bound to Ar^2);

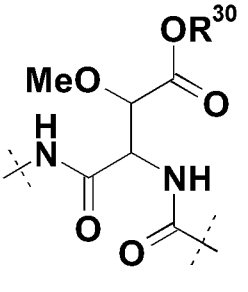
L^3 is a group of the following formula:



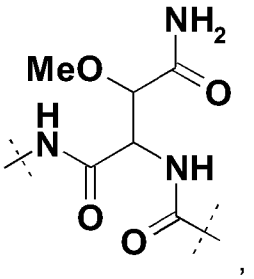
or



or



or



(wherein the NH group is bound to Ar^3), wherein R^{30} is a hydrogen atom or a C_{1-3}alkyl group (preferably, R^{30} is a hydrogen atom);

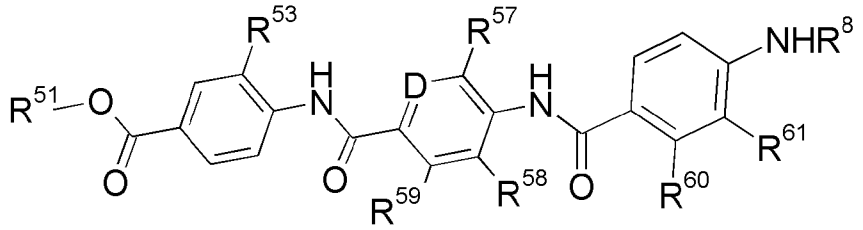
L^4 is absent or NHCO (wherein the nitrogen atom is bound to Ar^4);

R^1 is a group of formula $-NH_2$, $-NO_2$, $COOR^{11}$, or $-CONR^{12}R^{13}$; wherein R^{11}, R^{12} and R^{13} are independently a hydrogen atom or a C_{1-6} alkyl group (preferably, R^1 is a group of formula -COOH);

R^2 is a group of formula $-NH_2$, $-NO_2$, $COOR^{11a}$, or $-CONR^{12a}R^{13a}$; wherein R^{11a}, R^{12a} and R^{13a} are independently a hydrogen atom or a C_{1-6} alkyl group (preferably, R^2 is a group of formula $-NH_2$ or $-NO_2$);

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

2. A compound according to claim 1 of formula (VII)



(VII)

wherein

R^{51} is a hydrogen atom, or a C_{1-6} alkyl group;

R^{53} is F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

D is CR^{56};

R^{56} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{57} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

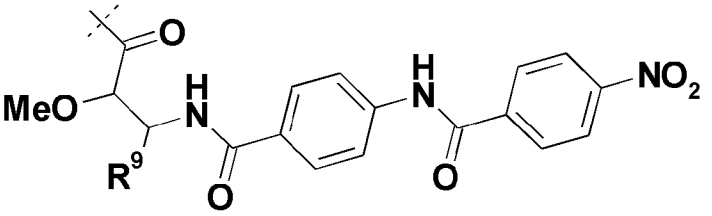
R^{58} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{59} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

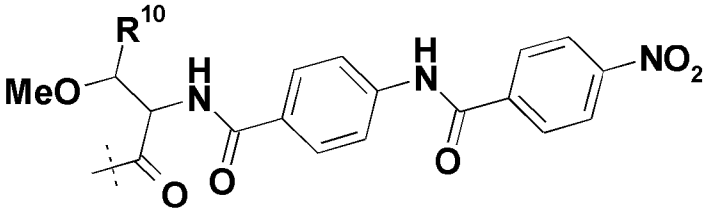
R^{60} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl;

R^{61} is a hydrogen atom, F, Cl, a hydroxy group, a C_{1-6} alkyl group or a group of formula -O-C_{1-6} alkyl; and

R^8 is a group of the following formula:



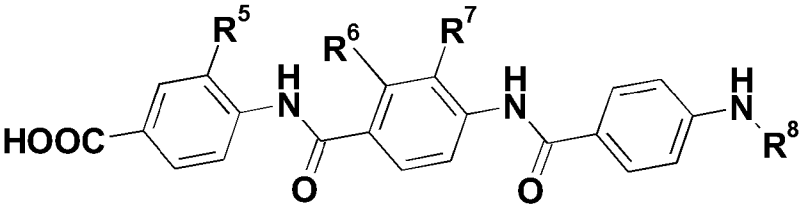
or



wherein R^9 is COOH or $CONH_2$ and R^{10} is COOH or $CONH_2$;

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

3. A compound according to claim 1 of formula (IV)



(IV)

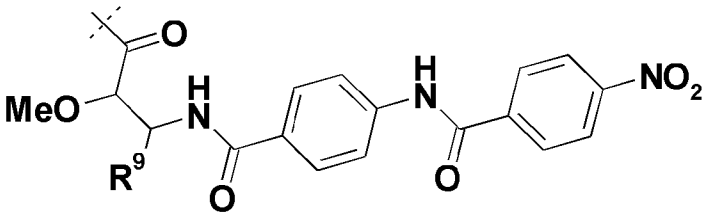
wherein

R^5 is a group of formula -O-C_{1-6} alkyl;

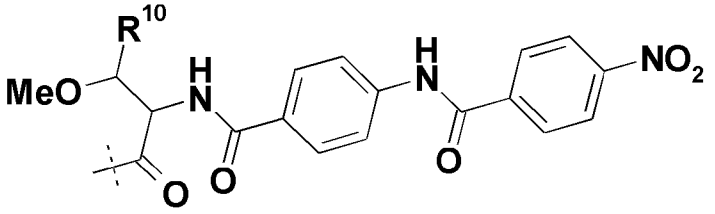
R^6 is a hydroxy group;

R^7 is a group of formula -O-C_{1-6} alkyl; and

R^8 is a group of the following formula:



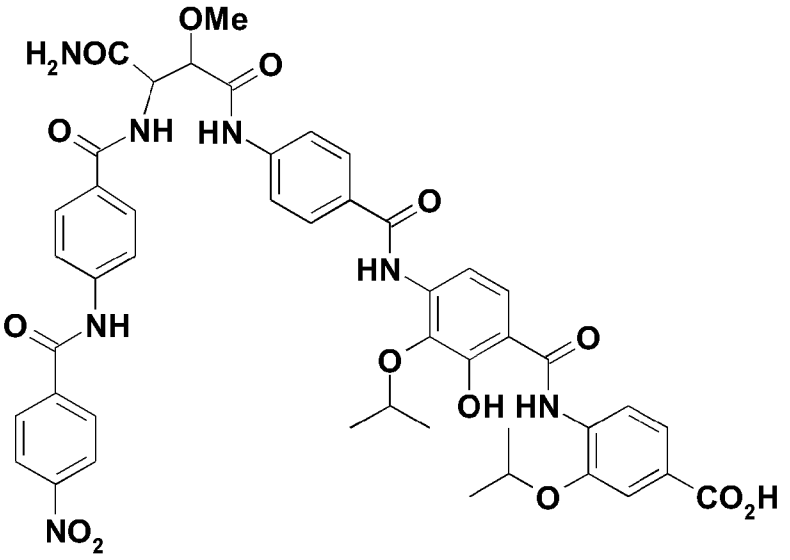
or



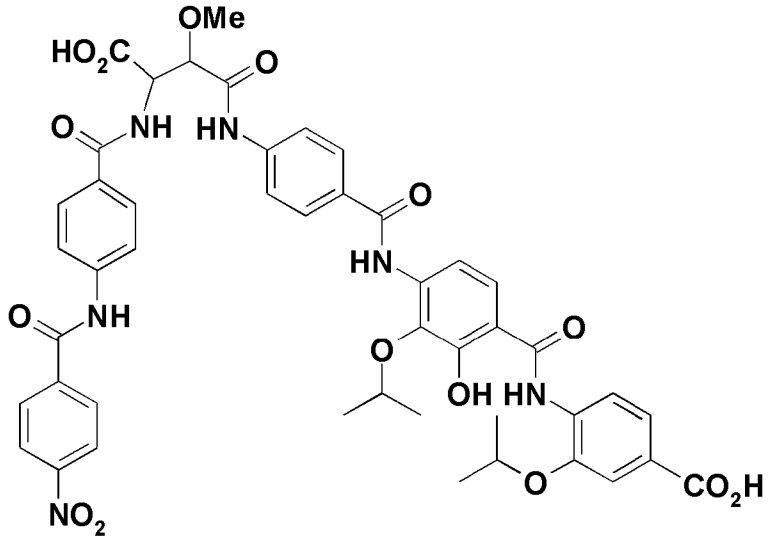
wherein R^9 is COOH or $CONH_2$ and R^{10} is COOH or $CONH_2$;

or a pharmaceutically acceptable salt, solvate or hydrate or a pharmaceutically acceptable formulation thereof.

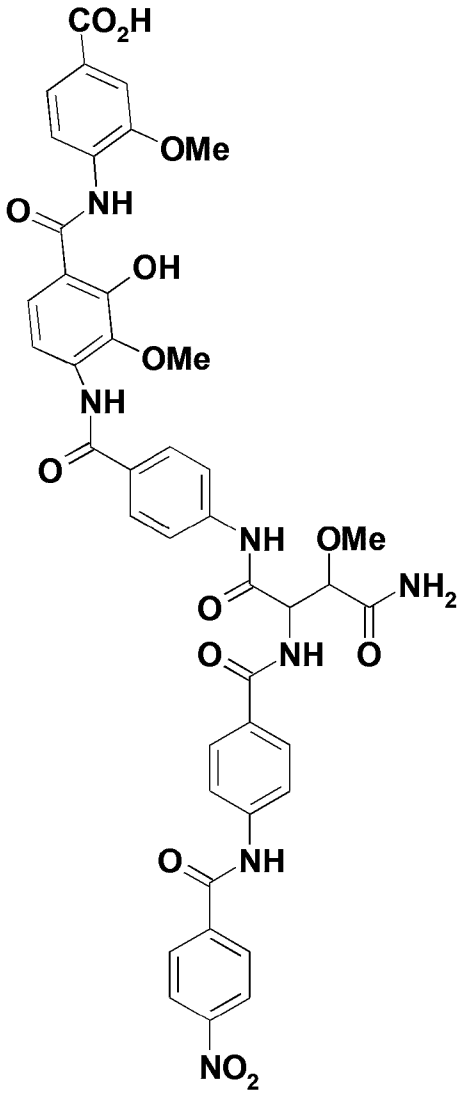
4. A compound according to claim 1 selected from:



Cystobactamide A (**1**);

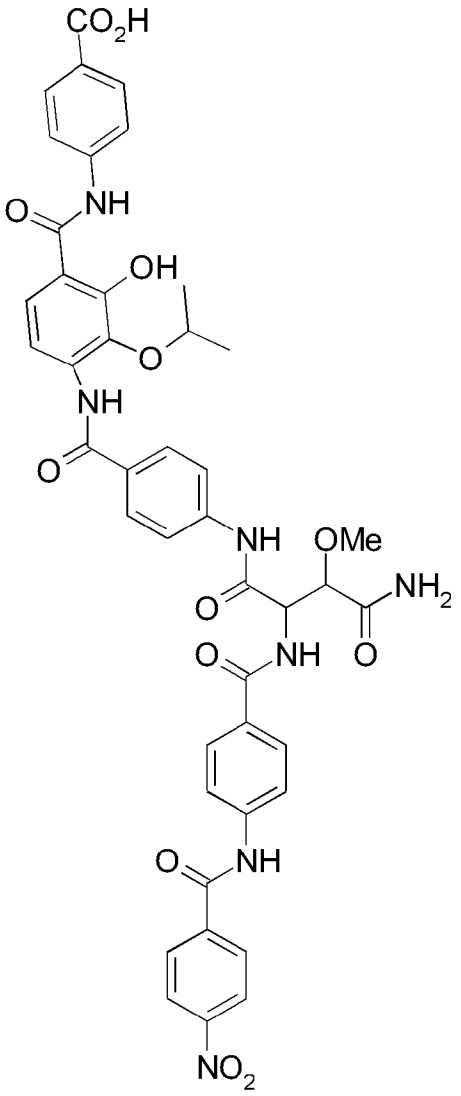


Cystobactamide B (**2**);

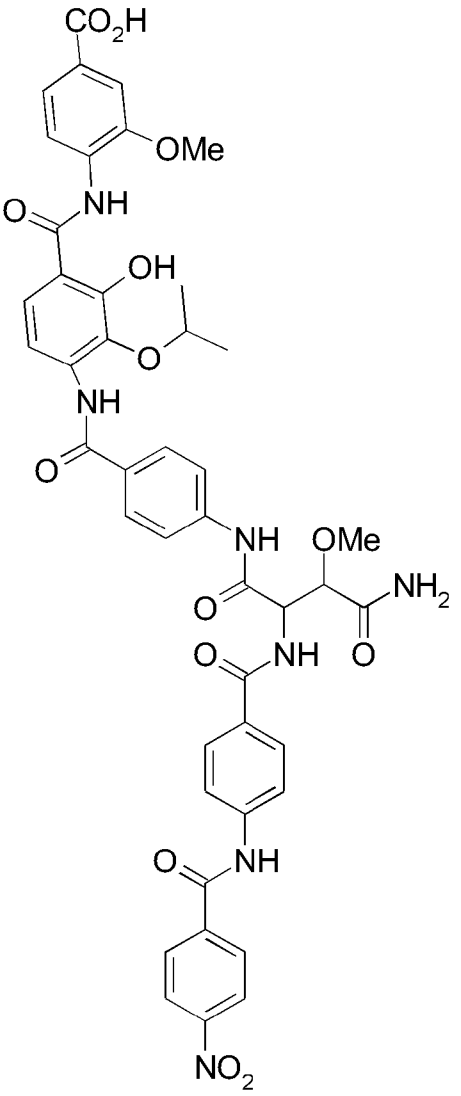


Cystobactamide D (**4**);

Date Reçue/Date Received 2020-11-06

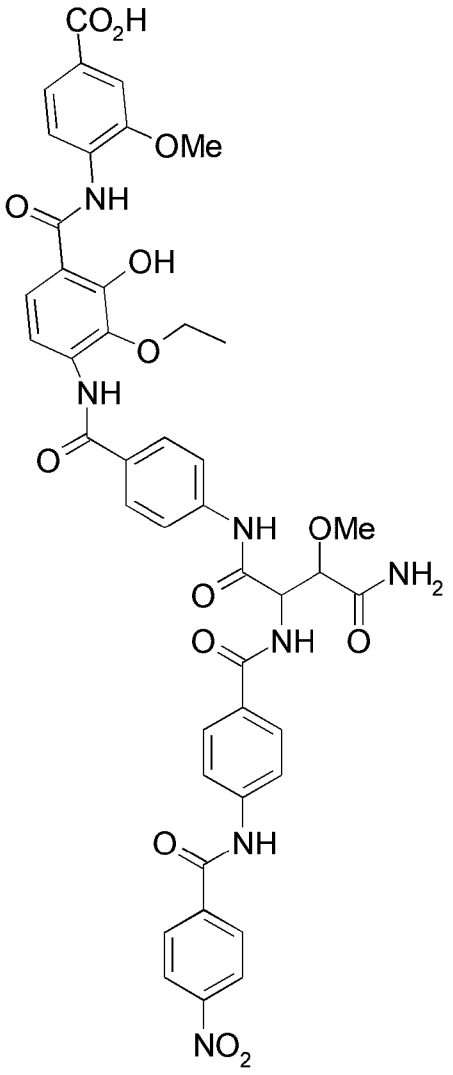


Cystobactamide F (**6**),



Cystobactamide G (**7**); and

Date Reçue/Date Received 2020-11-06



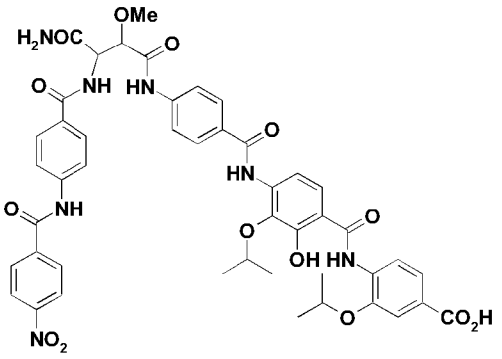
Cystobactamide H (**8**).

5. A pharmaceutical composition comprising a compound according to anyone of claims 1 to 4, and one or more carrier substances and/or one or more adjuvants.

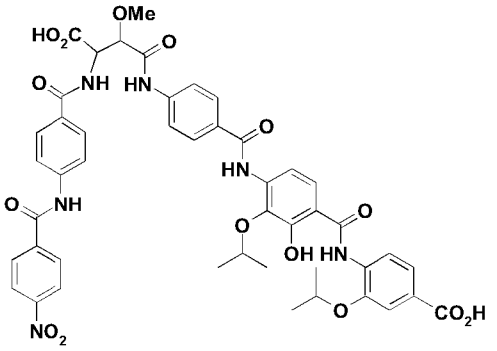
6. The compound according to anyone of claims 1 to 4 or the pharmaceutical composition according to claim 5 for use in the treatment or prophylaxis of bacterial infections.

Date Reçue/Date Received 2020-11-06

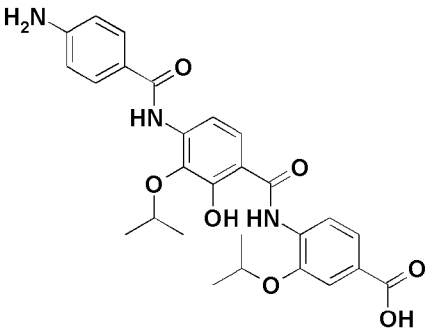
7. A recombinant biosynthesis cluster capable of synthesizing a cystobactamide selected from the group consisting of cystobactamide A, B, C, D, E, F, G and H:



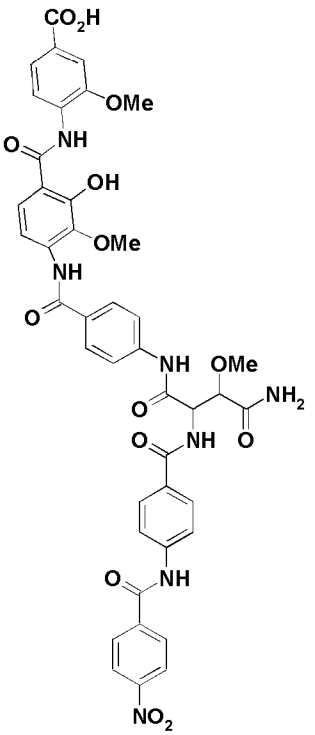
Cystobactamide A



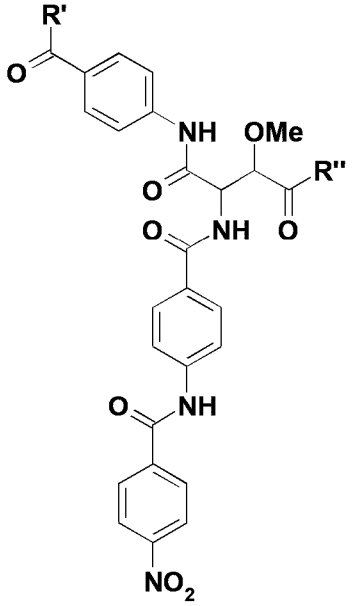
Cystobactamide B



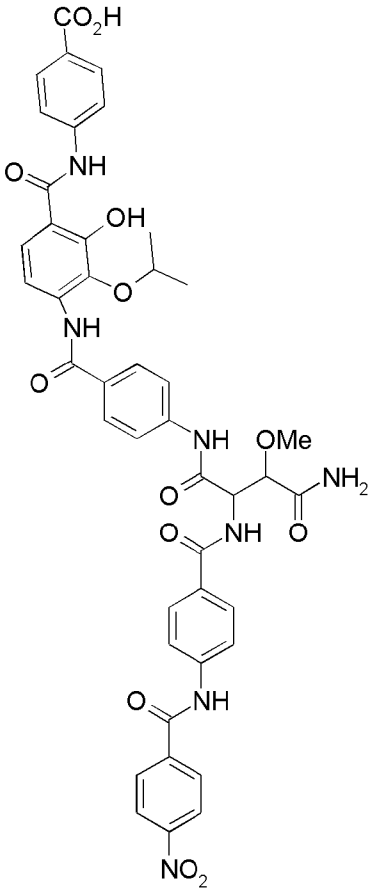
Cystobactamide C



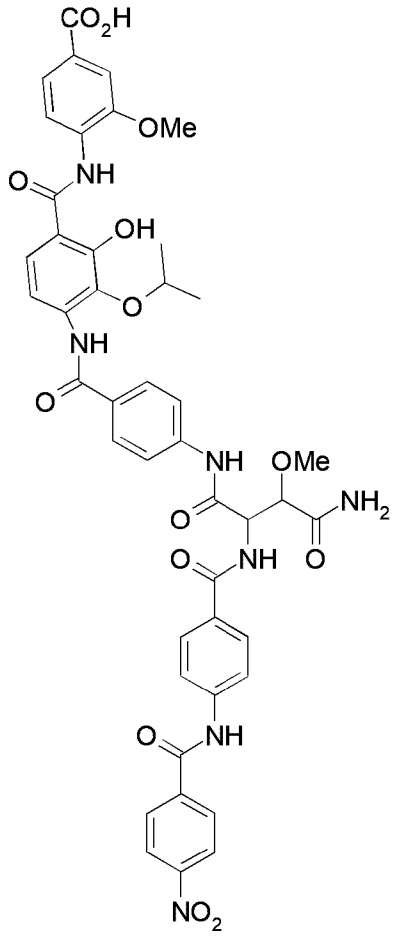
Cystobactamide D



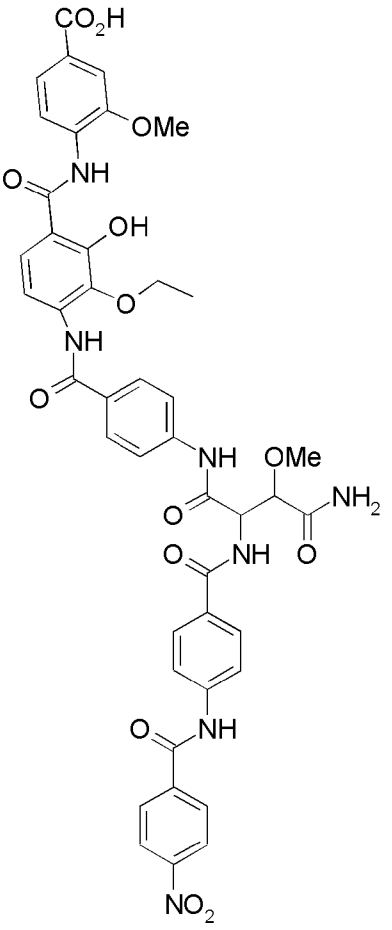
Cystobactamide E
(R' is NH_2 or OH and
R'' is NH_2 or OH);



Cystobactamide F



Cystobactamide G



Cystobactamide H,

wherein the cluster comprises all of the polypeptides according to SEQ ID NOs. 40 to 73.

8. An isolated, synthetic or recombinant nucleic acid comprising:
(i) a sequence encoding the cystobactamide biosynthesis cluster of claim 7, wherein the sequence has a sequence identity to the full-length sequence of SEQ ID NO. 1 from at least 85%, 90%, 95%, 96%, 97%, 98%, 98.5%, 99%, or 99.5% to 100%; or
(ii) a sequence completely complementary to the full length sequence of any nucleic acid sequence of (i).

9. A vector comprising at least one nucleic acid according to claim 8.

10. A recombinant host cell comprising at least one nucleic acid according to claim 8 or a vector according to claim 9.

11. A method for the preparation of a compound according to claim 4, the method comprising the steps of:
    (a) culturing *Cystobacter velatus* strain MCy8071 (DSM27004) or a recombinant host cell of claim 10; and
    (b) separating and retaining the compound from the culture broth.

12. Use of the compound according to anyone of claims 1 to 4, or the pharmaceutical composition according to claim 5, for the treatment or prophylaxis of bacterial infections.

13. Use of the compound according to anyone of claims 1 to 4, or the pharmaceutical composition according to claim 5, in the manufacture of a medicament for the treatment or prophylaxis of bacterial infections.

Date Reçue/Date Received 2020-11-06

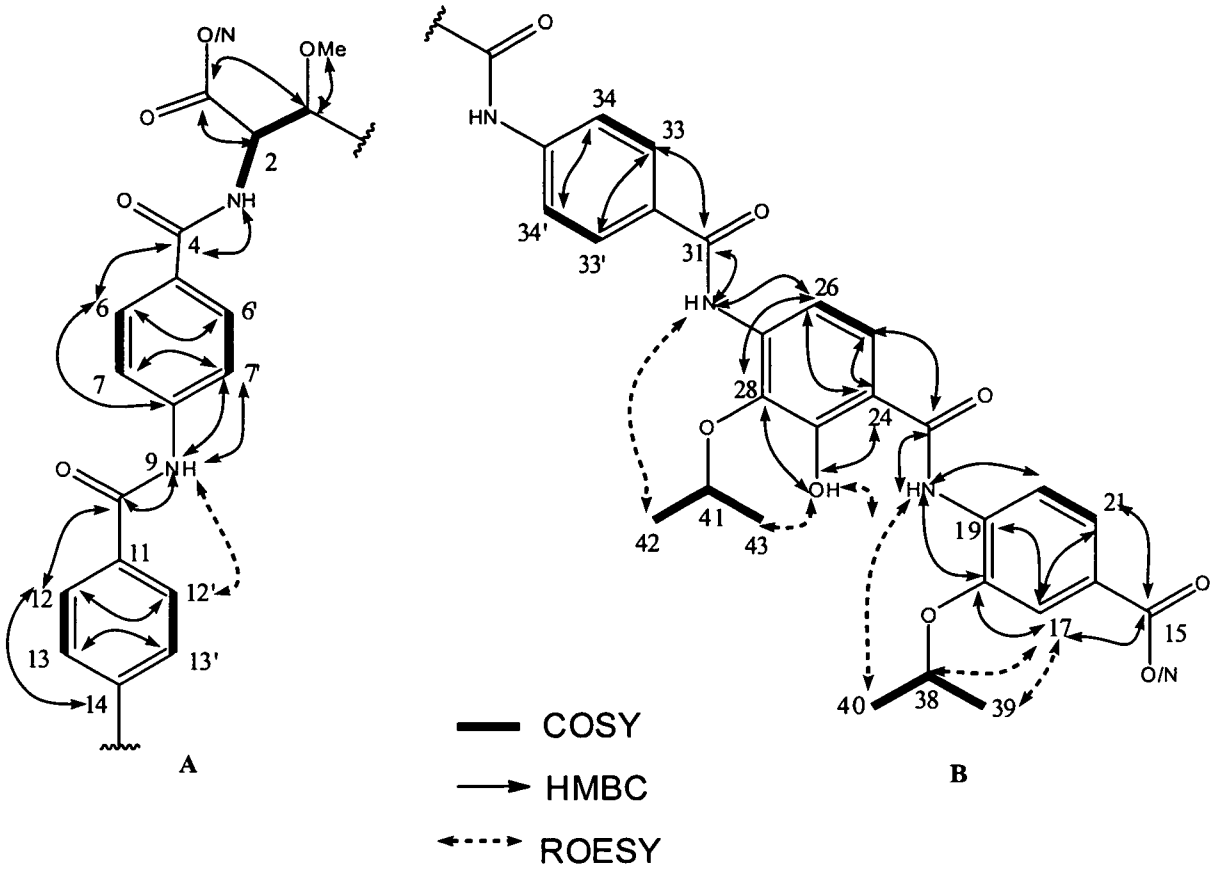

O/N
OMe
O
2
NH
O
4
6
6'
7
7'
9
NH
O
11
12
12'
13
13'
14
A
HN
34
33
34'
33'
31
O
HN
26
28
24
O
OH
O
41
42
43
HN
21
19
O
17
15
O/N
40
38
39
B
COSY
HMBC
ROESY

Figure 1

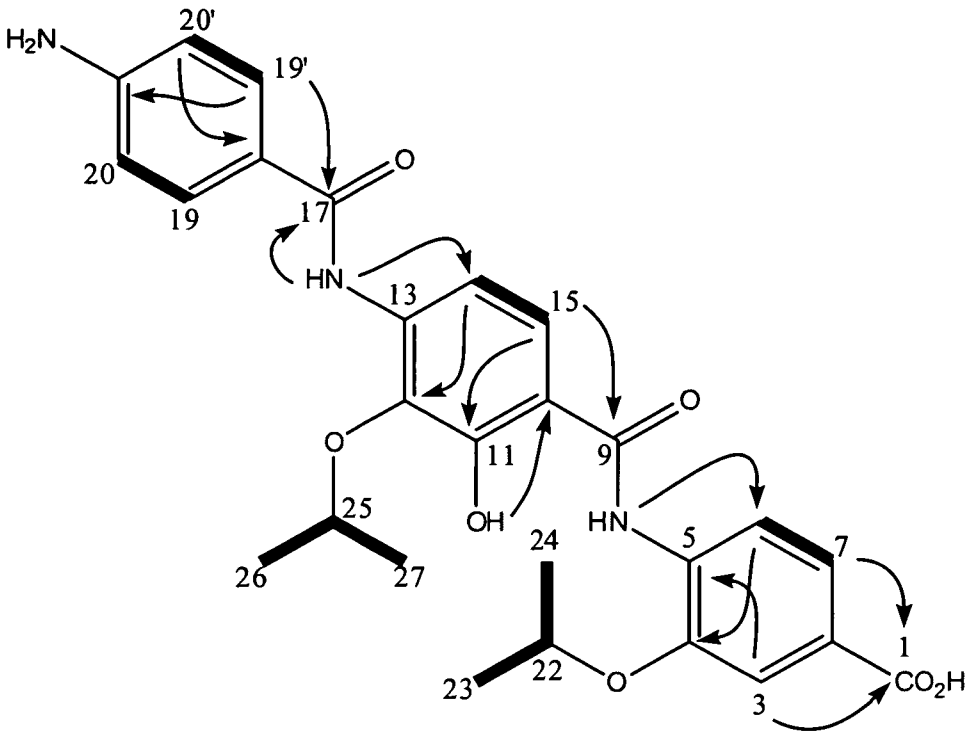

H2N
20'
19'
20
19
17
O
HN
13
15
O
O
11
9
25
OH
HN
5
7
24
26
27
1
22
23
O
CO2H
3

Figure 2

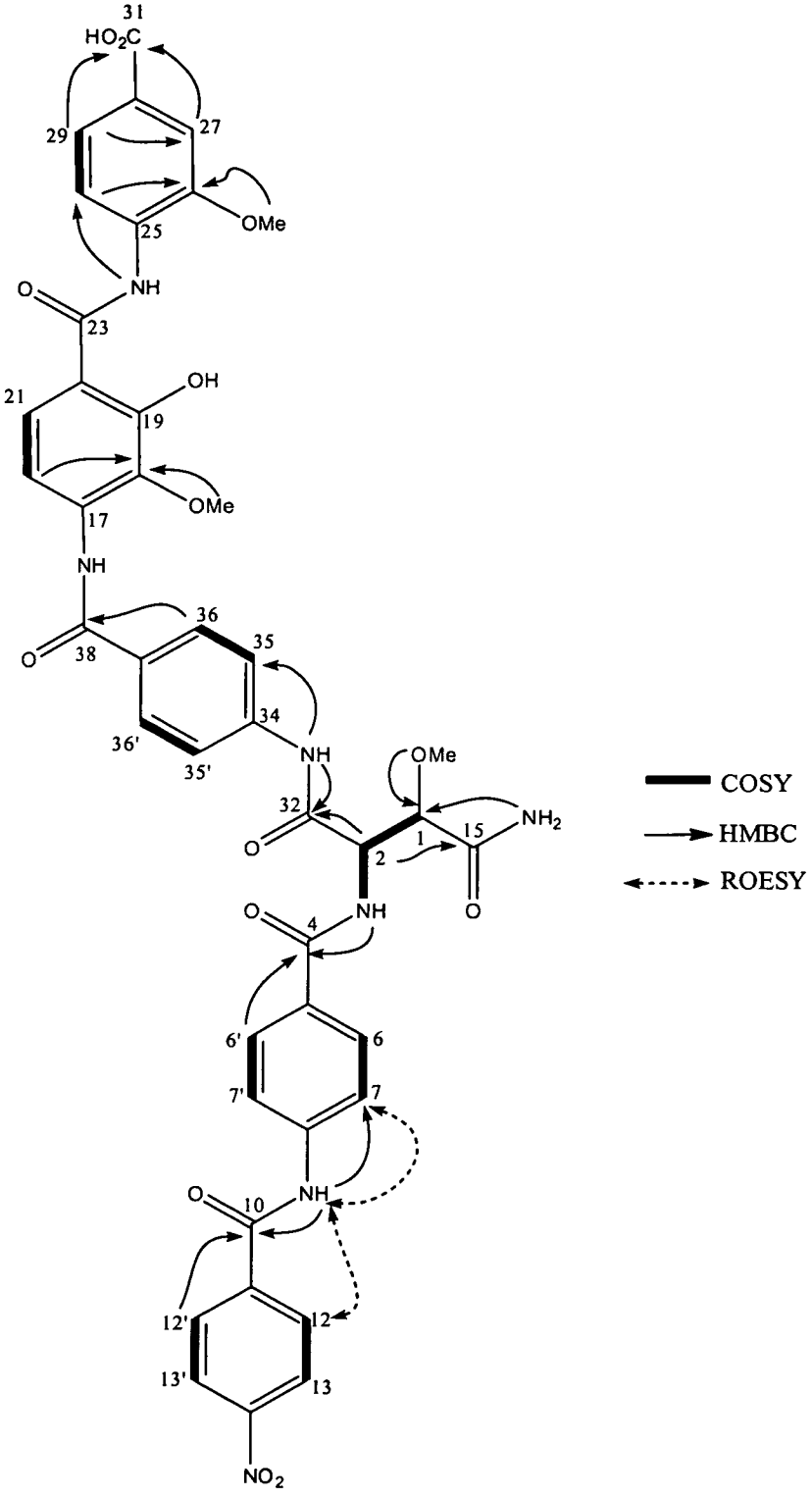

31
HO2C
29
27
OMe
25
NH
O
23
OH
21
19
OMe
17
NH
36
O
38
35
34
36'
35'
NH
OMe
32
NH2
O
1
15
2
O
NH
O
4
6'
6
7'
7
NH
O
10
12'
12
13'
13
NO2
COSY
HMBC
ROESY

Figure 3

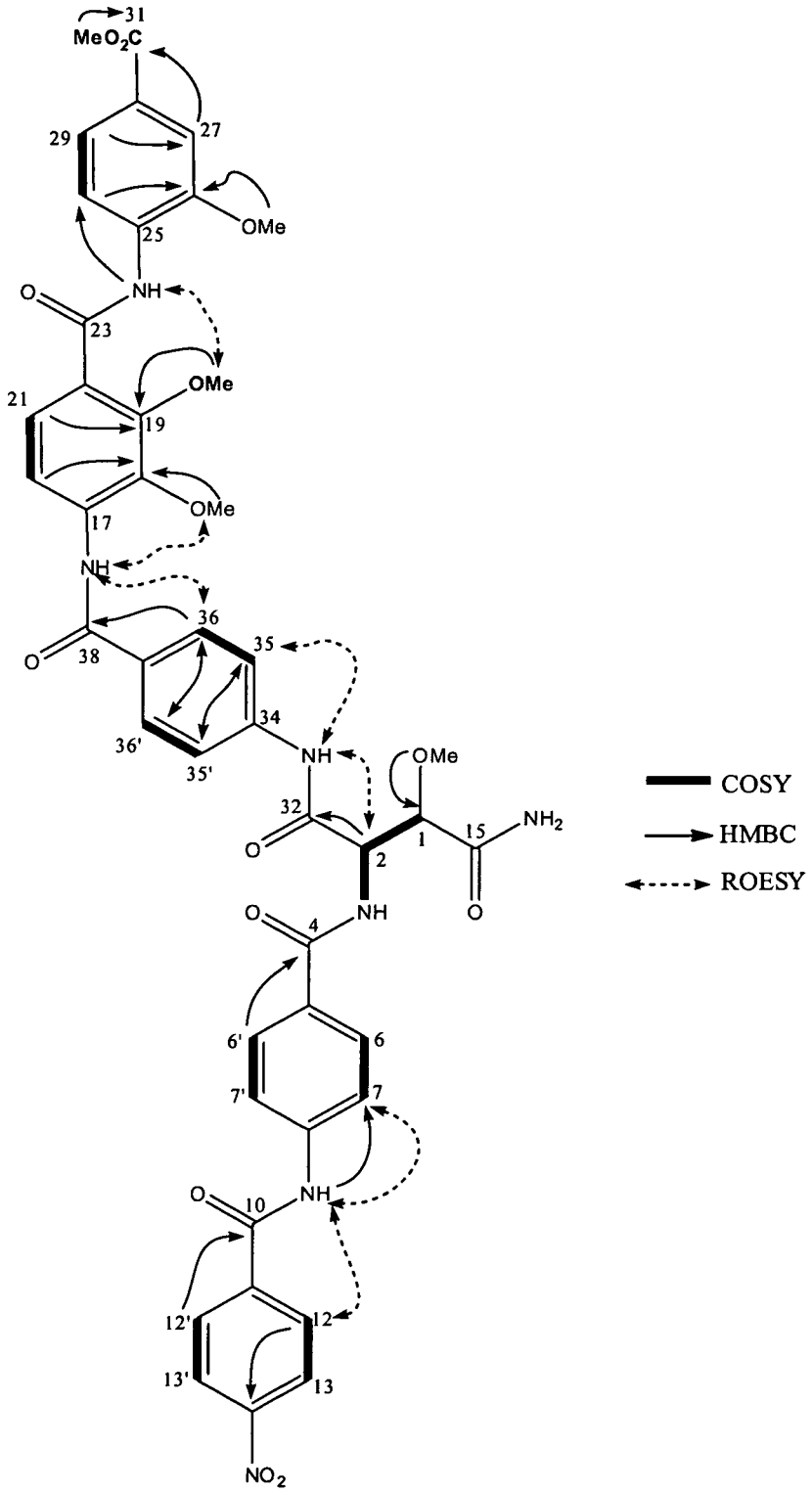

31
MeO2C
27
29
OMe
25
NH
O
23
OMe
21
19
OMe
17
NH
36
O
38
35
34
36'
35'
NH
OMe
32
O
1
15
NH2
2
O
NH
O
4
6'
6
7'
7
NH
O
10
12'
12
13'
13
NO2
COSY
HMBC
ROESY

Figure 4

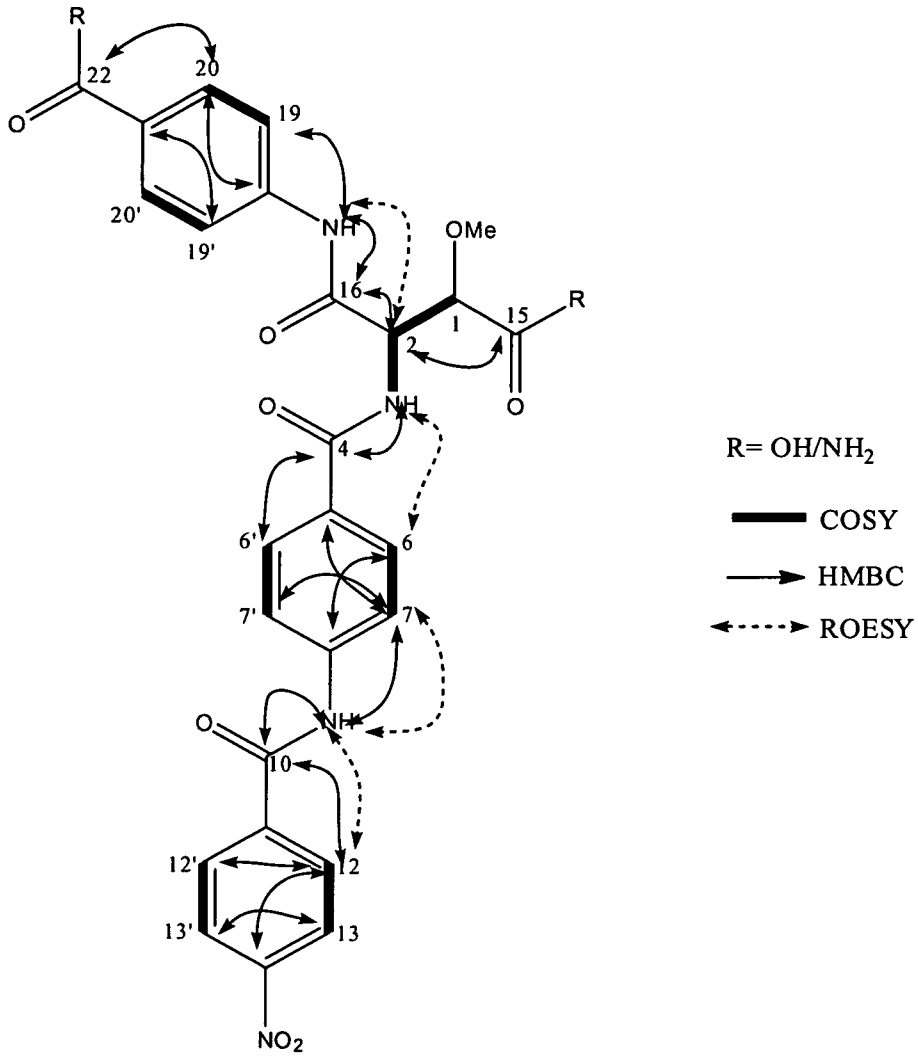

R
22
20
19
20'
19'
NH
OMe
16
15
R
1
2
O
O
NH
4
6'
6
7'
7
NH
O
10
12'
12
13'
13
NO2
R= OH/NH2
COSY
HMBC
ROESY

Figure 5

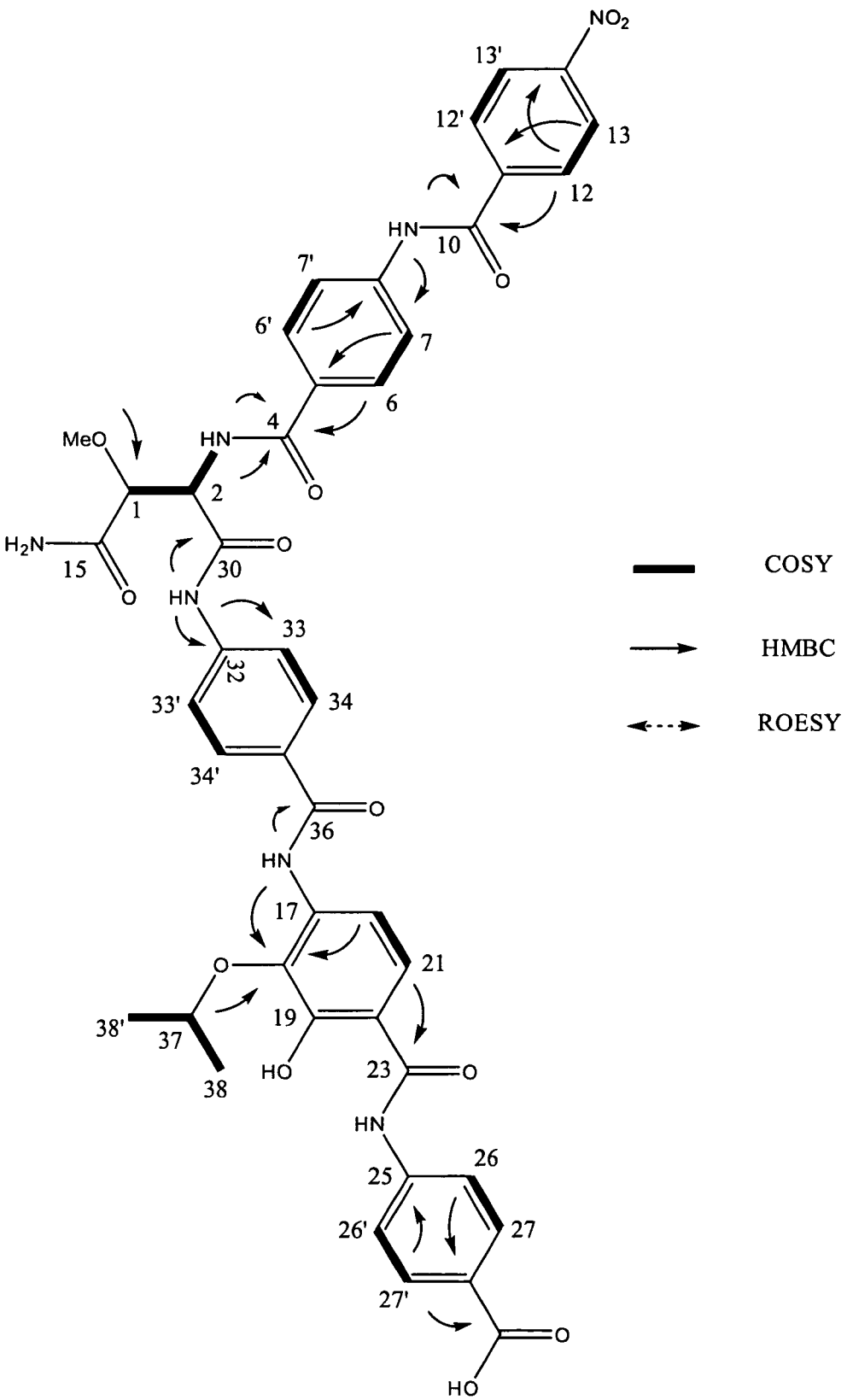

NO2
13'
12'
13
12
HN
10
O
7'
6'
7
6
4
HN
MeO
1
2
O
H2N
15
O
30
O
HN
33
32
33'
34
34'
O
36
HN
17
O
21
38'
37
19
38
HO
23
O
HN
26
25
26'
27
27'
O
HO
COSY
HMBC
ROESY

Figure 6

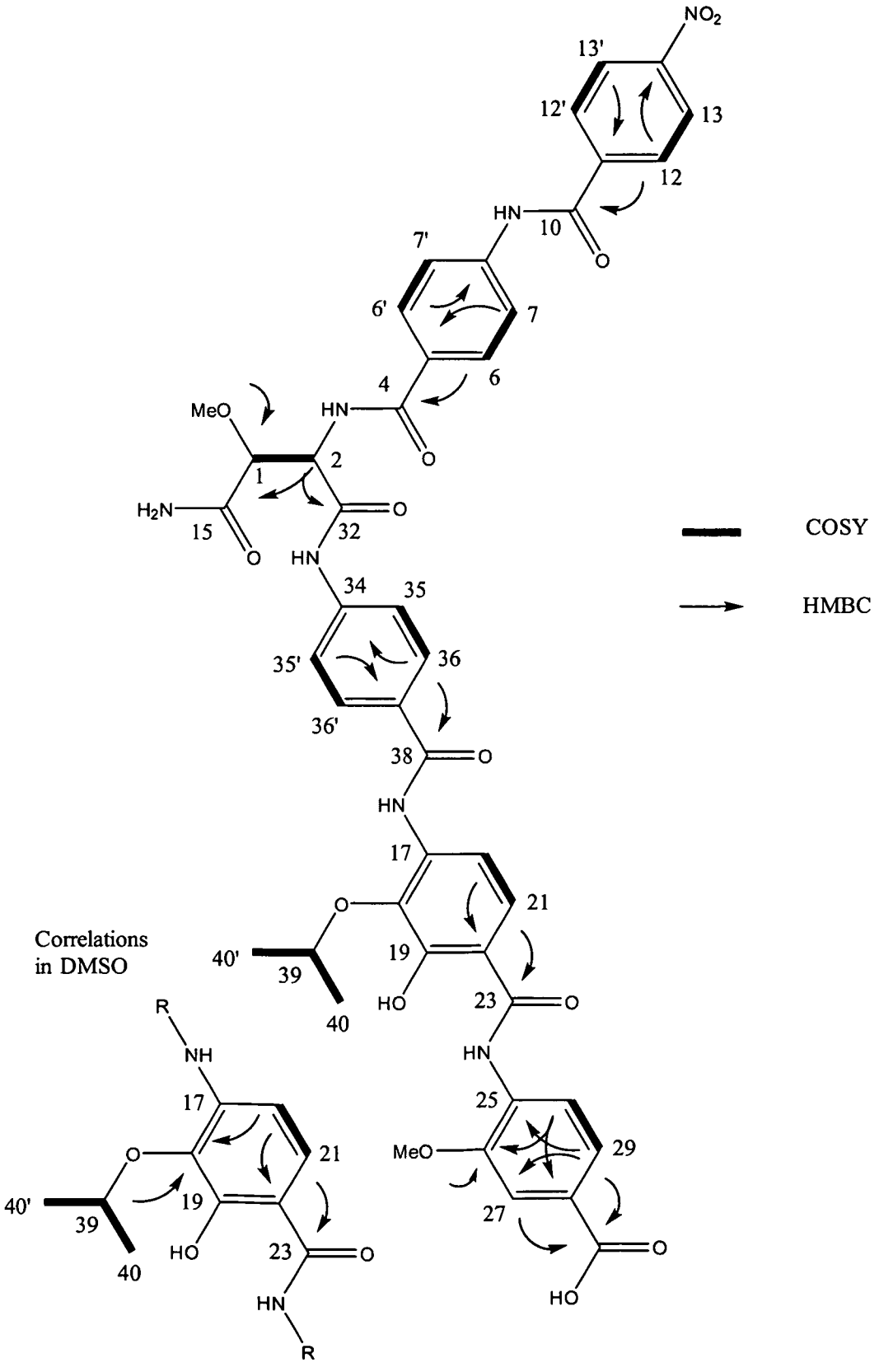

NO2
13'
12'
13
12
HN
10
O
7'
6'
7
6
4
HN
O
MeO
2
1
H2N
15
O
32
O
HN
34
35
35'
36
36'
38
O
HN
17
O
21
19
HO
23
O
HN
25
MeO
29
27
O
HO
40'
39
40
R
NH
17
O
21
19
HO
23
O
HN
R
40'
39
40
Correlations
in DMSO
COSY
HMBC

Figure 7

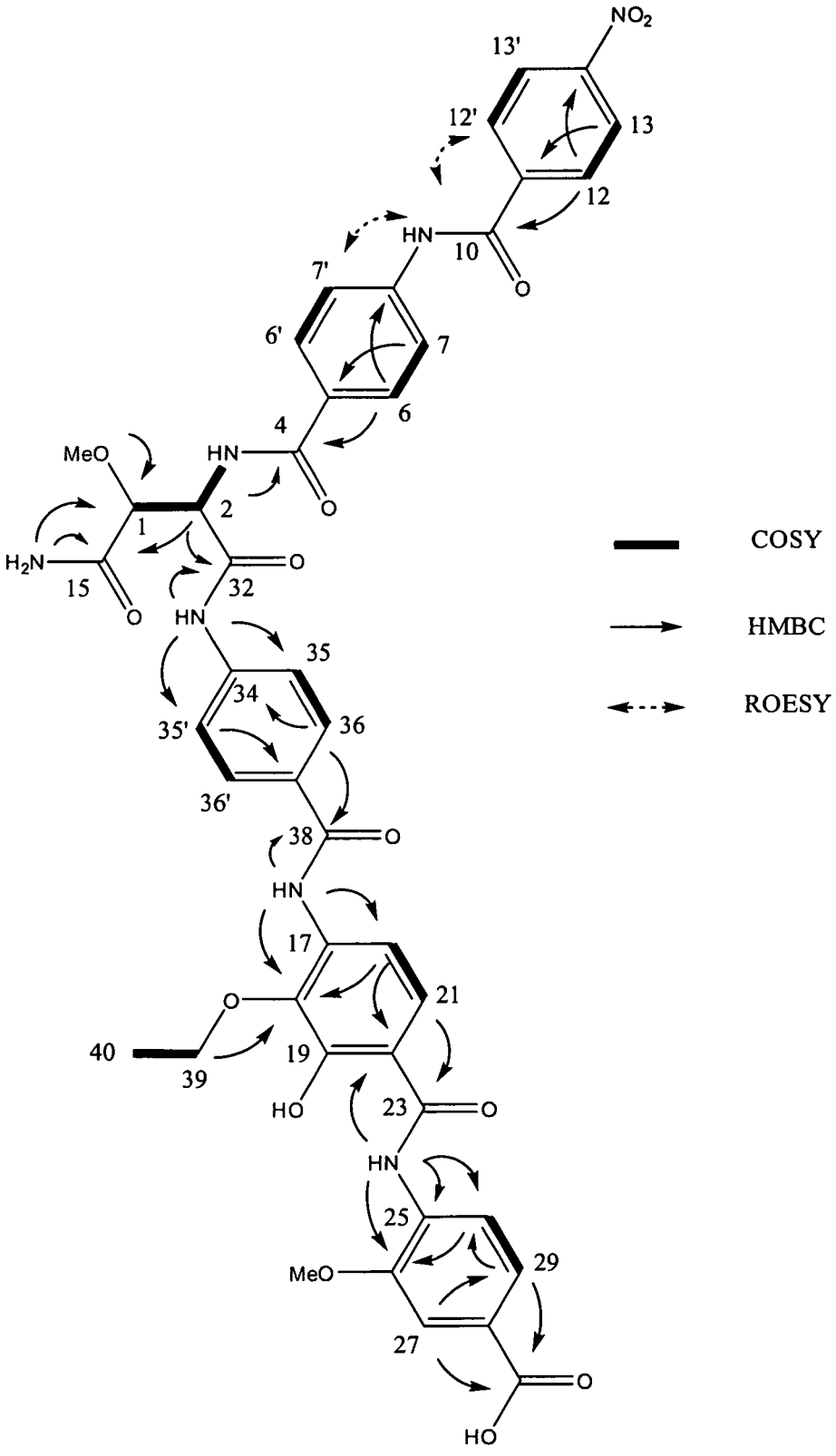

NO2
13'
12'
13
12
HN
10
O
7'
6'
7
6
4
HN
O
MeO
2
1
H2N
15
O
32
O
HN
35
34
35'
36
36'
38
O
HN
17
O
21
40
39
19
HO
23
O
HN
25
MeO
29
27
O
HO
COSY
HMBC
ROESY

Figure 8

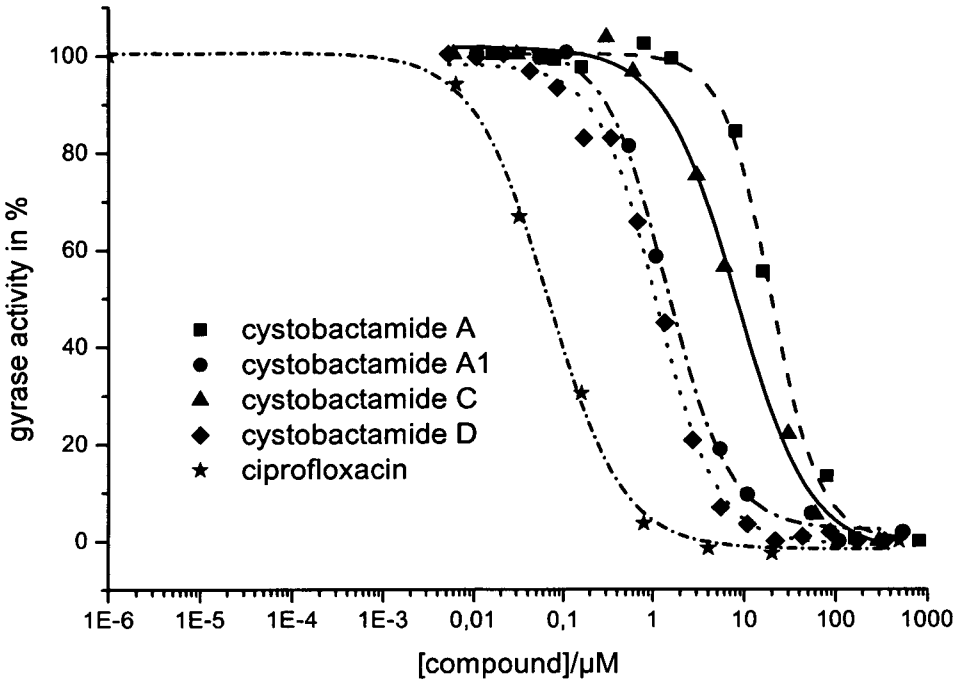

gyrase activity in %
100
80
60
40
20
0
cystobactamide A
cystobactamide A1
cystobactamide C
cystobactamide D
ciprofloxacin
1E-6
1E-5
1E-4
1E-3
0,01
0,1
1
10
100
1000
[compound]/µM

Figure 9a

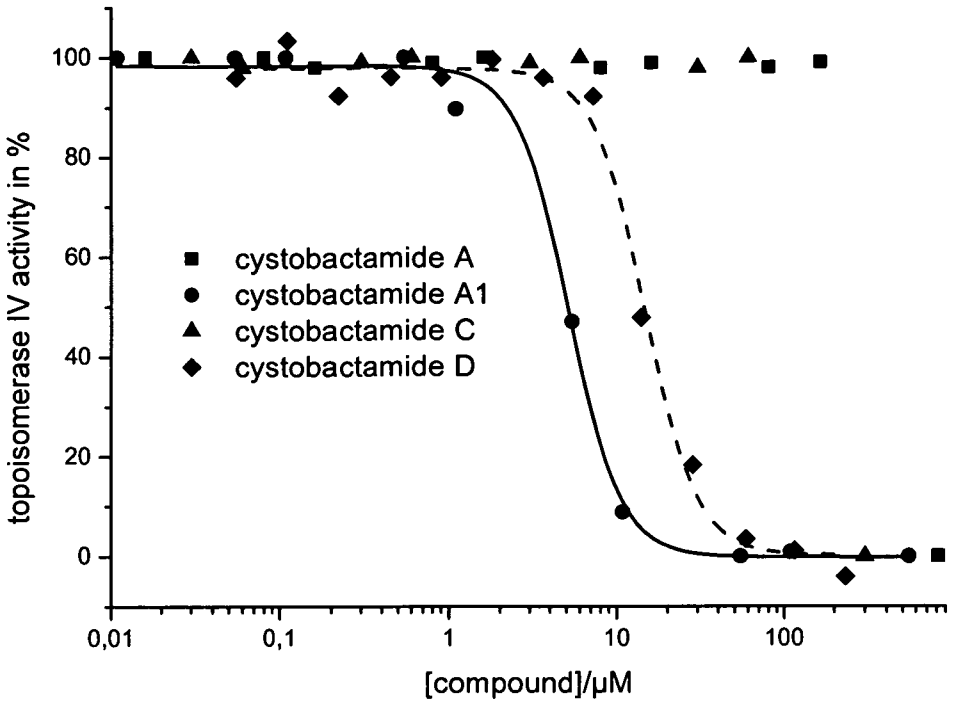

topoisomerase IV activity in %
100
80
60
40
20
0
0,01
0,1
1
10
100
[compound]/µM
cystobactamide A
cystobactamide A1
cystobactamide C
cystobactamide D

Figure 9b

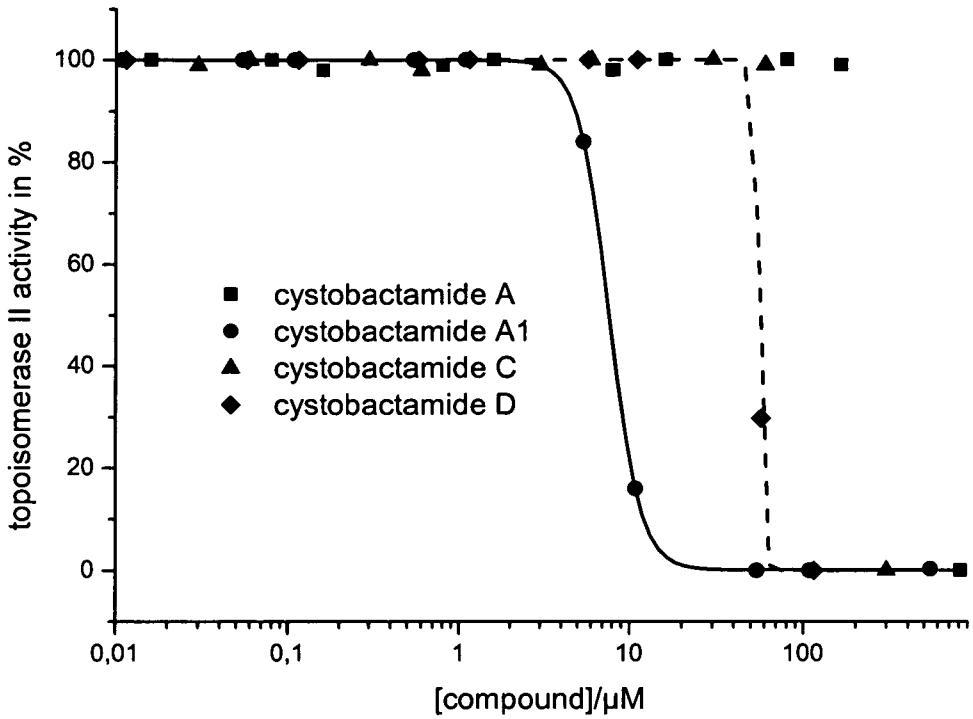

topoisomerase II activity in %
100
80
60
40
20
0
0,01
0,1
1
10
100
[compound]/µM
cystobactamide A
cystobactamide A1
cystobactamide C
cystobactamide D

Figure 9c

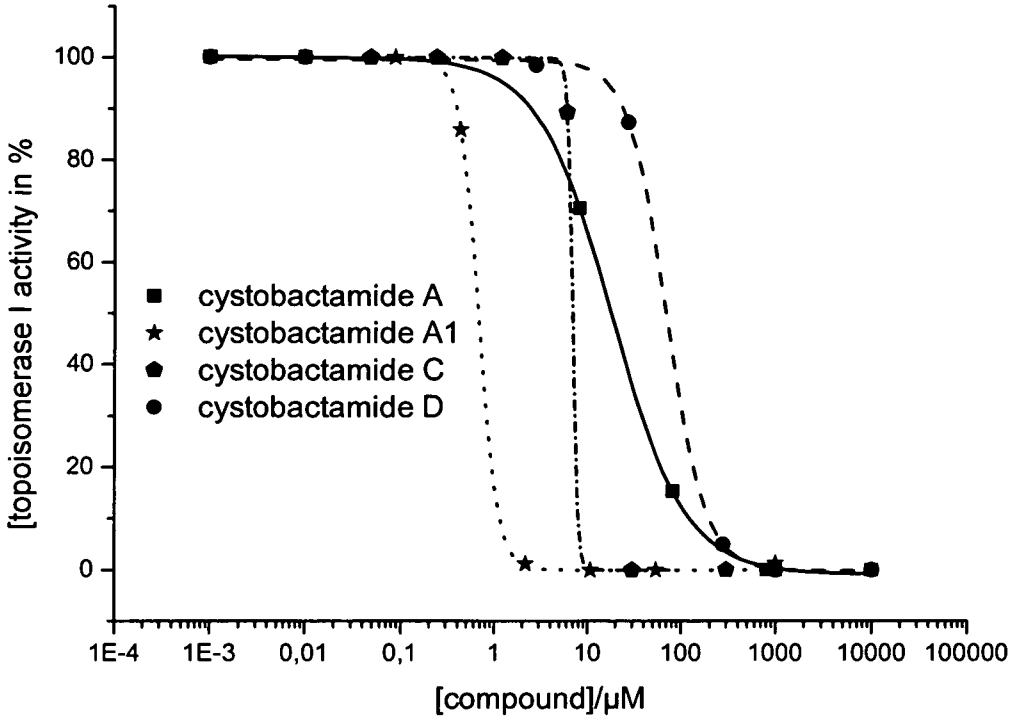
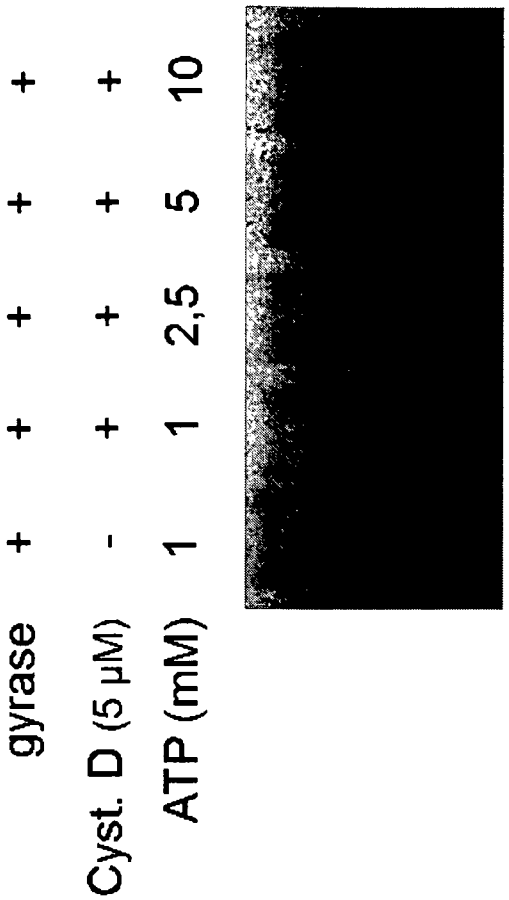

[topoisomerase I activity in %
100
80
60
40
20
0
cystobactamide A
cystobactamide A1
cystobactamide C
cystobactamide D
1E-4
1E-3
0,01
0,1
1
10
100
1000
10000
100000
[compound]/µM

Figure 9d



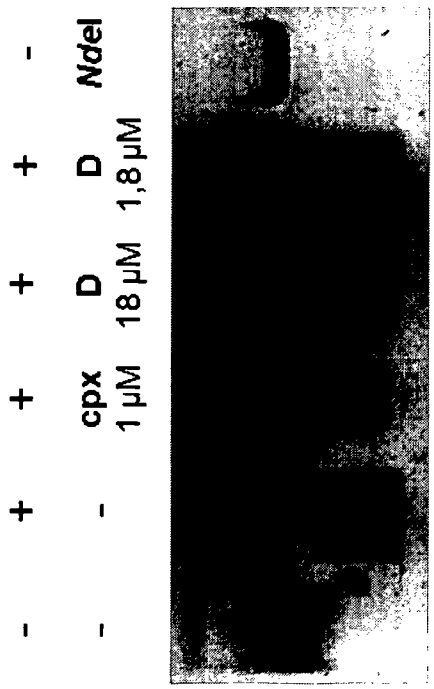


Figure 10 a and b

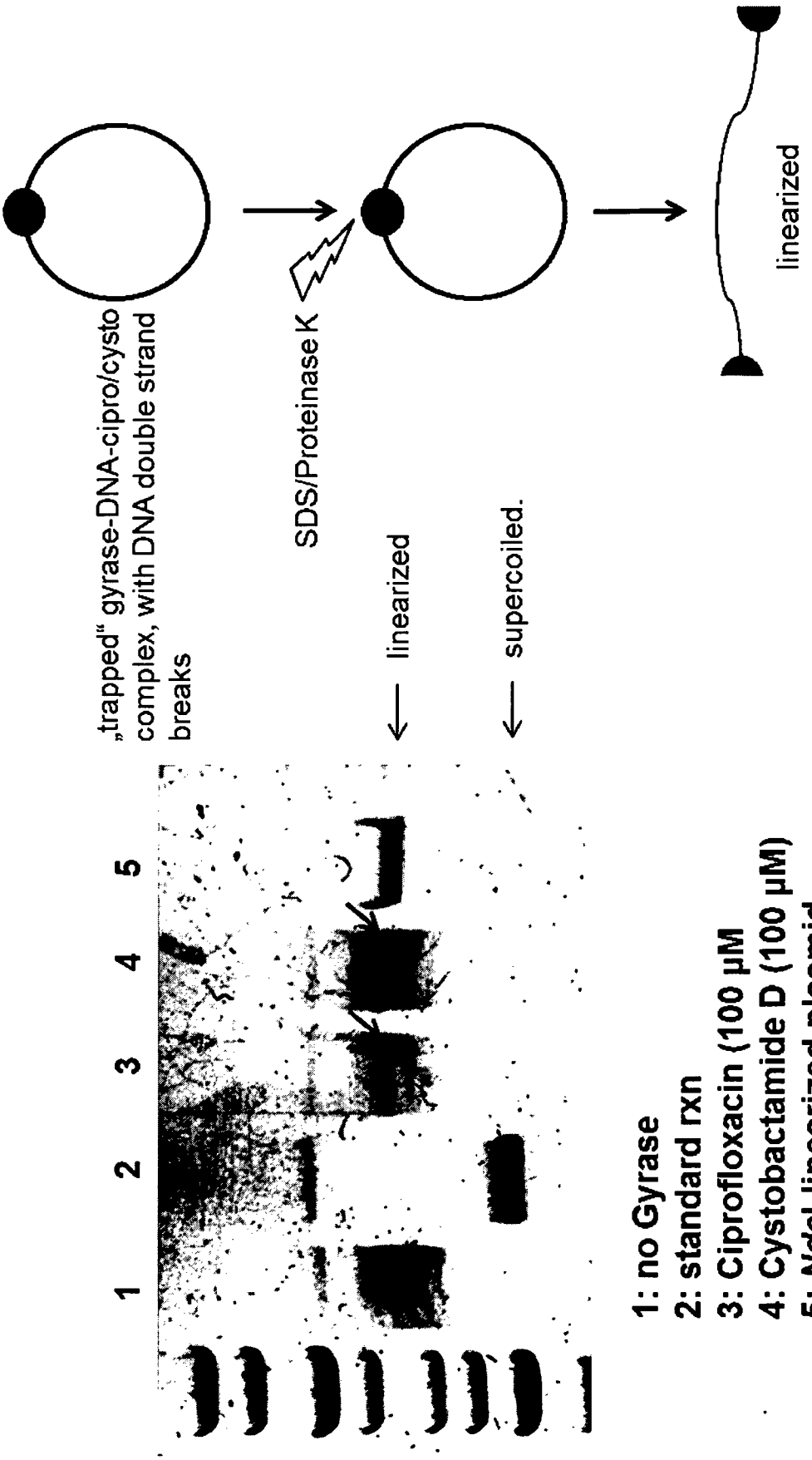

„trapped" gyrase-DNA-cipro/cysto complex, with DNA double strand breaks
SDS/Proteinase K
linearized
supercoiled.
linearized
1 2 3 4 5
1: no Gyrase
2: standard rxn
3: Ciprofloxacin (100 µM
4: Cystobactamide D (100 µM)
5: NdeI-linearized plasmid

Figure 11

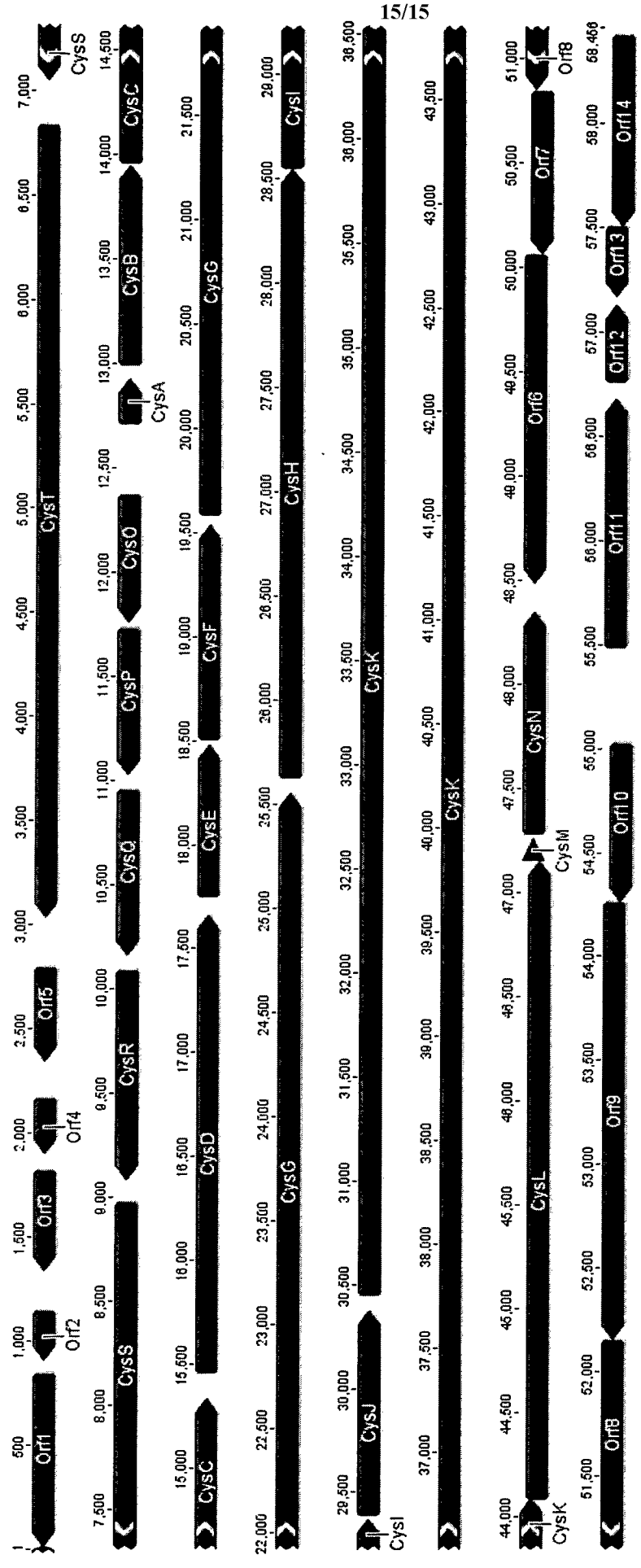

Orf1
Orf2
Orf3
Orf4
Orf5
CysT
CysS
CysC
CysR
CysS
CysA
CysB
CysC
CysD
CysO
CysP
CysE
CysF
CysG
CysC
CysG
CysH
CysI
CysJ
CysI
CysK
CysK
CysL
CysM
CysN
Orf6
Orf7
Orf8
Orf8
Orf9
Orf10
Orf11
Orf12
Orf13
Orf14

Figure 12