

New iridium-based complexes for ECL

Background of the Invention

The present invention relates to novel iridium-based Ir(III) luminescent complexes, conjugates comprising these complexes as a label and their application, e.g. in the electrochemiluminescence based detection of an analyte.

5 Electrogenated chemiluminescence (also called electrochemiluminescence and abbreviated ECL) is the process whereby species generated at electrodes undergo high-energy electron-transfer reactions to form excited states that emit light. The first detailed ECL studies were described by Hercules and Bard et al. in the mid-1960s. After about 40 years of study, ECL has now become a very powerful
10 analytical technique and is widely used in the areas of, for example, immunoassay, food and water testing, and biowarfare agent detection.

There is a tremendous number of compounds that appears to be of interest for use in organic light emitting devices (OLEDs). These compounds are appropriate for use in solid materials or may be dissolved in organic fluids. However, no
15 conclusion can be drawn regarding their utility in an aqueous medium as e.g., required for detection of an analyte from a biological sample.

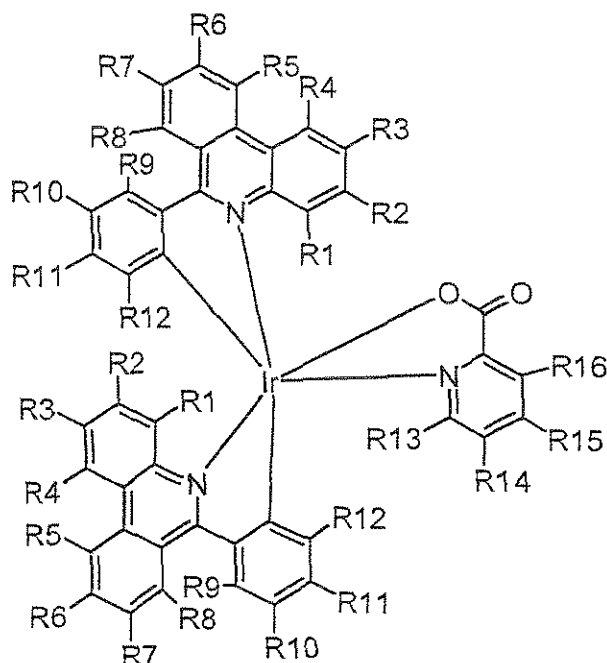
In general ECL-based detection methods are based on the use of water-soluble ruthenium complexes, comprising Ru(II+) as metal ion.

Despite significant improvements made over the past decades, still a tremendous
20 need exists for more sensitive electrochemiluminescence-based in vitro diagnostic assays.

It has now been surprisingly found that certain iridium-based Ir(III+) luminescent complexes, represent very promising labels for future high sensitive ECL-based detection methods.

Summary

The present disclosure, in a first aspect, discloses an iridium-based chemiluminescent compound of Formula I



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wherein R1-R16 is hydrogen, halide, cyano- or nitro-group, amino, alkylamino, substituted alkylamino, arylamino, substituted arylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or R17, wherein R17 is aryl, substituted aryl, alkyl, substituted alkyl branched alkyl, substituted branched alkyl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy,

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substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or,

wherein within R1-R12 or/and within R13-R16, respectively, two adjacent Rs can form an aromatic ring or a substituted aromatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or,

wherein within R1-R12 or/and within R13-R16, respectively, two adjacent Rs can form an aliphatic ring or a substituted aliphatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate and

wherein at least one of R13-R16 is -Q-Y, wherein Q represents a linker and Y is a functional group.

The present disclosure, in a second aspect, also discloses a conjugate comprising the above compound and covalently bound thereto an affinity binding agent.

The present disclosure further relates to the use of a compound or of a conjugate as disclosed in the present disclosure for performing a luminescence measurement or an electrochemiluminescence reaction in an aqueous solution, especially, in a electro-chemiluminescent device or electrochemiluminescent detection system.

The present disclosure, in a third aspect, relates to the use of a compound as defined above or of a conjugate as defined above for performing an electrochemiluminescence reaction in an aqueous solution.

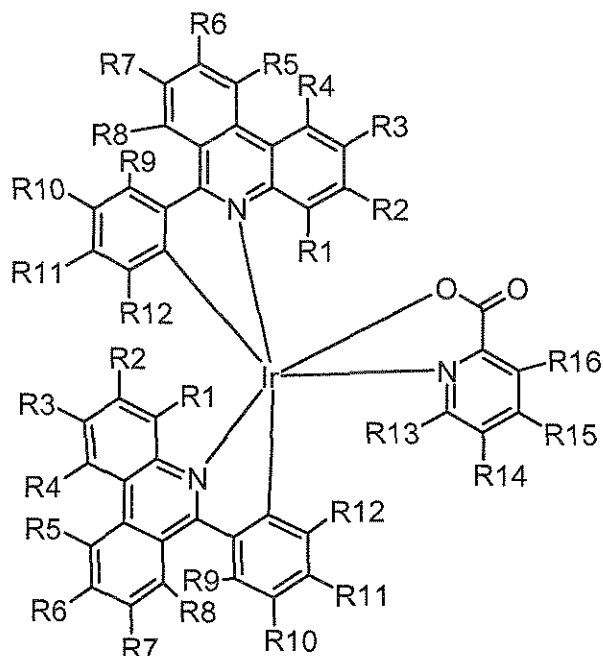
The present disclosure, in a fourth aspect, relates to the use of a compound as defined above or of a conjugate as defined above in an electrochemiluminescence based detection method.

The present disclosure, in a fifth aspect, relates to the use of a compound as defined above or of a conjugate as defined above in the detection of an analyte.

Further the present disclosure, in a sixth aspect, discloses a method for measuring an analyte by an in vitro method, the method comprising the steps of (a) providing a sample suspected or known to comprise the analyte, (b) contacting said sample with a conjugate according to the present disclosure under conditions appropriate for formation of an analyte conjugate complex, and (c) measuring the complex formed in step (b) and thereby obtaining a measure of the analyte.

Detailed Description of the Invention

The present invention relates to an iridium-based chemiluminescent compound of Formula I



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wherein R1-R16 is hydrogen, halide, cyano- or nitro-group, amino, alkylamino, substituted alkylamino, arylamino, substituted arylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfinio, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or R17, wherein R17 is aryl, substituted aryl, alkyl, substituted alkyl branched alkyl, substituted branched alkyl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo,

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5 sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or, wherein within R1-R12 or/and within R13-R16, respectively, two adjacent Rs can form an aromatic ring or a substituted aromatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like
10 amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or, wherein within R1-R12 or/and within R13-R16,
15 respectively, two adjacent Rs can form an aliphatic ring or a substituted aliphatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate and wherein at least one of R13-R16 is -Q-Y, wherein Q represents a linker and Y is a functional group.

20 In one embodiment at least one of R1 to R16 of the compound according to Formula I is substituted by at least one hydrophilic group.

In one embodiment preferred substituents for substituted alkyloxy are ethylenoxy chains comprising 1-40 ethylenoxy units, or comprising 1-20 ethylenoxy units or comprising 1-10 ethylenoxy units.

25 Preferred hydrophilic groups are amino, alkylamino, with alkyl meaning a linear chain such as methyl, ethyl, propyl, butyl pentyl chain or a branched alkyl chain such as isopropyl, isobutyl, tert. butyl, preferably a linear alkyl chain such as methyl or ethyl, substituted alkylamino, this contains one or two for example a branched or linear chains bound to the N-atom, which are substituted by an additional hydrophilic group such as hydroxyl or sulfo, preferably this substituted
30 alkylamino contains two hydroxypropyl or hydroxyethyl residues, arylamino, with aryl referring to an aromatic residue, such as phenyl, or naphthyl, preferably phenyl, substituted arylamino, with aryl as defined above and an additional residue formed by a hydrophilic group, alkylammonium, with alkyl as defined above and preferably being a trimethylammonium residue or triethylammonium residue,
35 substituted alkylammonium, carboxy, carboxylic acid ester, preferably an alkyl

5 ester such as methyl or ethyl ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy with alkyl and substituted alkyl being as defined above or aryloxy or substituted aryloxy with aryl and substituted aryl being as defined above, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfinio, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate.

10 Preferably such hydrophilic group is selected from amino, alkylamino, substituted alkylamino, arylamino, substituted arylamino, alkylammonium, substituted alkylammonium, carboxy, hydroxy, sulfo, sulfeno, sulfonamide, sulfoxide, sulfodioxide and phosphonate, where applicable, each preferably as defined in the above paragraph.

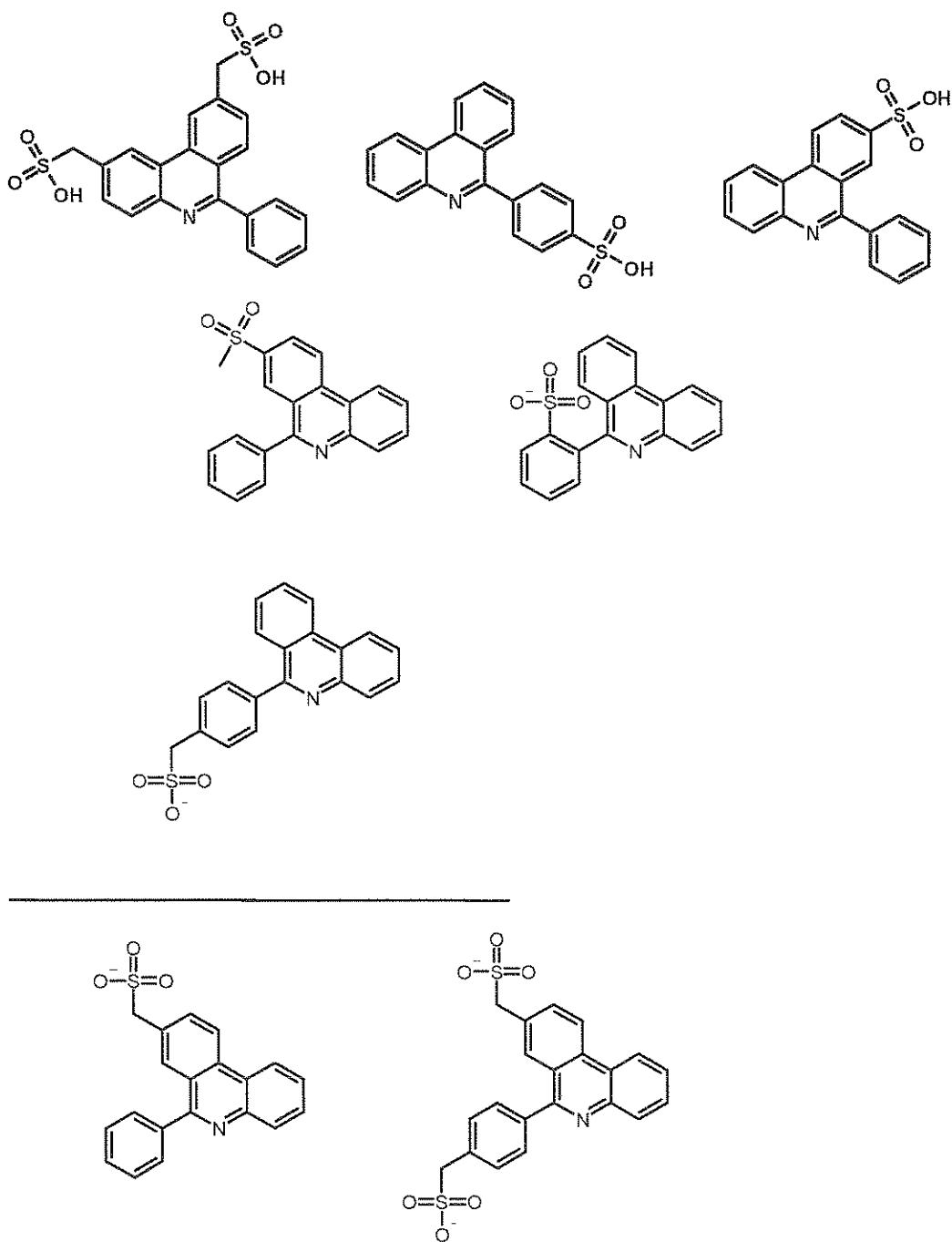
In a further embodiment the hydrophilic group is selected from sulfo, sulfonamide, sulfodioxide.

In one embodiment at least one of the groups R1 to R12 of Formula I is a sulfo group.

15 In one embodiment at least one of R1 to R12 of the phenylphenantridine residues comprised in Formula I is substituted by at least one hydrophilic group.

In one embodiment the phenylphenantridine residues comprised in Formula I are selected from the below given substituted phenylphenantridines.

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In the compound according to the present invention the linker Q preferably has a backbone length of between 1 and 20 atoms. With other words, the shortest connection between the pyridyl ring of Formula I and the functional group Y consists of 1 to 20 atoms. In one embodiment, the linker Q in the electrochemiluminescent complex of this invention is a straight or branched saturated, unsaturated, unsubstituted, substituted C1-C20 alkyl chain, or a C1-C20 arylalkyl chain (wherein e.g. a phenylen ring accounts for a length of four carbon

atoms), or a 1 to 20 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S, or a 1 to 20 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S comprising at least one aryl, heteroaryl, substituted aryl or substituted heteroaryl group (wherein e.g. a phenylen ring accounts for a length of four atoms).
5 In one embodiment the linker Q in a compound according to the present invention is a saturated C1-C12 alkyl chain, or a C1-C12 arylalkyl chain, or a 1 to 12 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S, or a 1 to 12 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S comprising at
10 least one aryl, heteroaryl, substituted aryl or substituted heteroaryl group (wherein e.g. a phenylen ring accounts for a length of four atoms).

In one embodiment the functional group Y comprised in the iridium-based complex according to the present invention is selected from the group consisting of
15 carboxylic acid, N-hydroxysuccinimide ester, amino group, halogen, sulfhydryl, maleimido, alkynyl, azide, and phosphoramidite.

A conjugate comprising an iridium-based electrochemiluminescent compound of Formula I as disclosed and defined herein above and covalently bound thereto a biological substance. Examples of suitable biological substances are cells, viruses,
20 subcellular particles, proteins, lipoproteins, glycoproteins, peptides, polypeptides, nucleic acids, peptidic nucleic acids (PNA), oligosaccharides, polysaccharides, lipopoly-saccharides, cellular metabolites, haptens, hormones, pharmacological substances, alkaloids, steroids, vitamins, amino acids and sugars.

In one embodiment the biological substance of a conjugate according to the present invention, i.e., covalently bound to a compound according Formula I is an affinity
25 binding agent. As the skilled artisan will appreciate in a conjugate according to the present invention the functional group Y of the compound according to Formula I has been used to form a covalent bond with a group on the affinity binding agent. In case an affinity binding reagent would not in itself contain an appropriate group
30 for binding or reacting with the group Y, such group can be easily introduced into the affinity binding agent by relying on well-established procedures.

Not wishing to be limited further, but in the interest of clarity, the affinity binding agent may comprise any of the following; an antigen, a protein, an antibody, biotin or biotin analogue and avidin or streptavidin, sugar and lectin, an enzyme, a

polypeptide, an amino group, a nucleic acid or nucleic acid analogue and complementary nucleic acid, a nucleotide, a polynucleotide, a peptide nucleic acid (PNA), a polysaccharide, a metal-ion sequestering agent, receptor agonist, receptor antagonist, or any combination thereof. For example, the affinity binding agent can
5 be one partner of a specific binding pair, where the other partner of said binding pair is associated with or is the target on a cell surface or an intracellular structure.

Preferably an affinity binding agent is, a partner or member of an affinity binding pair, or as it is also called by the skilled artisan, a partner or member of a specific binding pair.

10 An affinity binding agent has at least an affinity of 10^7 l/mol to its target, e.g. one member of a specific binding pair, like an antibody, to the other member of the specific binding pair, like its antigen. An affinity binding agent preferably has an affinity of 10^8 l/mol or even more preferred of 10^9 l/mol for its target.

15 In one embodiment the present invention relates to a conjugate wherein the affinity binding agent is selected from the group consisting of antigen, antibody, biotin or biotin analogue, avidin or streptavidin, sugar, lectin, nucleic acid or nucleic acid analogue and complementary nucleic acid, receptor and ligand.

In one embodiment the present invention relates to a conjugate wherein the affinity binding agent is selected from the group consisting of antibody, biotin or biotin
20 analogue, avidin or streptavidin, and nucleic acid.

In one embodiment the conjugate according to the present invention comprises covalently linked a compound according to Formula I as disclosed and defined herein above and an affinity binding agent that either is an oligonucleotide or an antibody.

25 Biotin analogues are aminobiotin, iminobiotin or desthiobiotin.

The term "oligonucleotide" or "nucleic acid" as used herein, generally refers to short, generally single stranded, polynucleotides that comprise at least 8 nucleotides and at most about 1000 nucleotides. In a preferred embodiment an oligonucleotide will have a length of at least 9, 10, 11, 12, 15, 18, 21, 24, 27 or 30
30 nucleotides. In a preferred embodiment an oligonucleotide will have a length of no more than 200, 150, 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides.

The term oligonucleotide is to be understood broadly and includes DNA and RNA as well as analogues and modification thereof.

A nucleic acid analogue may for example contain a substituted nucleotide carrying a substituent at the standard bases deoxyadenosine (dA), deoxyguanosine (dG),
 5 deoxycytosine (dC), deoxythymidine (dT), deoxyuracil (dU). Examples of such substituted nucleobases are: 5-substituted pyrimidines like 5 methyl dC, aminoallyl dU or dC, 5-(aminoethyl-3-acrylimido)-dU, 5-propinyl-dU or -dC, 5 halogenated -dU or -dC; N substituted pyrimidines like N4-ethyl-dC; N substituted purines like N6-ethyl-dA, N2-ethyl-dG; 8 substituted purines like 8-[6-amino)-hex-1-yl]-8-amino-dG or -dA, 8 halogenated dA or dG, 8 -alkyl dG or dA; and 2 substituted
 10 dA like 2 amino dA.

A nucleic acid analogue may contain a nucleotide or a nucleoside analogue. I.e. the naturally occurring nucleobases can be exchanged by using nucleobase analogs like 5-Nitroindol d riboside; 3 nitro pyrrole d riboside, deoxyinosine (dI),
 15 deoxyxanthosine (dX); 7 deaza -dG, -dA, -dI or -dX; 7-deaza-8-aza -dG, -dA, -dI or -dX; 8-aza -dA, -dG, -dI or -dX; d Formycin; pseudo dU; pseudo iso dC; 4 thio dT; 6 thio dG; 2 thio dT; iso dG; 5-methyl-iso-dC; N8-linked 8-aza-7-deaza-dA; 5,6-dihydro-5-aza-dC; and etheno-dA or pyrrolo-dC. As obvious to the skilled artisan, the nucleobase in the complementary strand has to be selected in such manner that
 20 duplex formation is specific. If, for example, 5-methyl-iso-dC is used in one strand (e.g. (a)) iso dG has to be in the complementary strand (e.g. (a')).

In a nucleic acid analogue the oligonucleotide backbone may be modified to contain substituted sugar residues, sugar analogs, modifications in the internucleoside phosphate moiety, and/or be a PNA.

25 An oligonucleotide may for example contain a nucleotide with a substituted deoxy ribose like 2'-methoxy, 2'-fluoro, 2'-methylseleno, 2'-allyloxy, 4'-methyl dN (wherein N is a nucleobase, e.g., A, G, C, T or U).

Sugar analogs are for example Xylose; 2',4' bridged Ribose like (2'-O, 4'-C methylene)- (oligomer known as LNA) or (2'-O, 4'-C ethylene)- (oligomer known
 30 as ENA); L-ribose, L- d-ribose, hexitol (oligomer known as HNA); cyclohexenyl (oligomer known as CeNA); altritol (oligomer known as ANA); a tricyclic ribose analog where C3' and C5' atoms are connected by an ethylene bridge that is fused to a cyclopropane ring (oligomer known as tricycloDNA); glycerin (oligomer known as GNA); Glucopyranose (oligomer known as Homo DNA); carbaribose

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(with a cyclopentan instead of a tetrahydrofuran subunit); hydroxymethyl-morpholin (oligomers known as morpholino DNA)

A great number of modification of the internucleosidic phosphate moiety are also known not to interfere with hybridization properties and such backbone
5 modifications can also be combined with substituted nucleotides or nucleotide analogs. Examples are phosphorothioate, phosphordithioate, phosphoramidate and methylphosphonate oligonucleotides.

PNA (having a backbone without phosphate and d-ribose) can also be used as a DNA analog.

10 The above mentioned modified nucleotides, nucleotide analogs as well as oligonucleotide backbone modifications can be combined as desired in an oligonucleotide in the sense of the present invention.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g.
15 bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research,
20 diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues
25 of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily,
30 however, isolated antibody will be prepared by at least one purification step.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond,

- while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light-chain and heavy-chain variable domains.
- 10 The “variable region” or “variable domain” of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as “VH.” The variable domain of the light chain may be referred to as “VL.” These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.
- 15 The term “variable” refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVRs) both in
- 20 the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs
- 25 in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but
- 30 exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The “light chains” of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, 5 IgG3, IgG4, IgA1, and IgA2. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and Mol. Immunology, 4th ed., W.B. Saunders, Co. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one 10 or more other proteins or peptides.

The terms "full-length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

15 "Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

20 Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields a F(ab')₂ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

25 "Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy 30 chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three

HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

5 The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody-hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

15 "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, e.g., Plueckthun, In: The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenberg and Moore (eds.), Springer-Verlag, New York (1994) pp. 269-315.

20 The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 0404 097; WO 1993/01161; Hudson, P.J. et al., Nat. Med. 9 (2003) 129-134; and Holliger, P. et al., PNAS USA 90 (1993) 6444-6448. Triabodies and tetrabodies are also described in Hudson, P.J. et al., Nat. Med. 9 (2003) 129-134.

30 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal

antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target-binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal-antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal-antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

As mentioned, the compounds and conjugates as disclosed herein have quite favorable properties. For example the disclosed compounds or conjugates, respectively, show a high ECL efficiency. This high efficiency is also present if the corresponding measurements are performed in an aqueous system as compared to many, many ECL-labels that only have shown high ECL-efficiency when analyzed in an organic solvent. E.g., many OLED dyes usually are analyzed in acetonitrile and either are not soluble in an aqueous solution or, if soluble, due not show efficient electrochemiluminescence in an aqueous solution.

In one preferred embodiment the present invention relates the use of a compound or of a conjugate, respectively, as disclosed in the present invention for performing an electrochemiluminescence reaction in an aqueous solution. An aqueous solution is any solution comprising at least 90% water (weight by weight). Obviously such aqueous solution may contain in addition ingredients like buffer compounds, detergents and for example tertiary amines like tripropylamine as electron donor in the ECL reaction.

In one embodiment the present invention relates to the use of a compound or of a conjugate, respectively, as disclosed in the present invention in an electrochemiluminescence based detection method.

In one embodiment the present invention relates the use of a compound or of a conjugate, respectively, as disclosed in the present invention in the detection of an analyte.

5 An analyte according to the present invention may be any inorganic or organic molecule, including any biological substance of interest. Examples of suitable biological substances that represent an analyte in the sense of the present invention are cells, viruses, subcellular particles, proteins, lipoproteins, glycoproteins, peptides, polypeptides, nucleic acids, oligosaccharides, polysaccharides, lipopoly-
10 saccharides, cellular metabolites, haptens, hormones, pharmacological substances, alkaloids, steroids, vitamins, amino acids and sugars.

The analyte may be selected from the group consisting of a polypeptide, a carbohydrate, and an inorganic or organic drug molecule.

A polypeptide or protein is a molecule that is essentially composed of amino acids and that has at least two amino acids linked by peptidic linkage. In case the analyte
15 of interest to be investigated in a method disclosed here, the polypeptide preferably will consist of at least 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, and 30 to up to about 10,000 amino acids. Preferably the polypeptide will contain from 5 to 2,000, also preferred from 10 to 1,000 amino acids.

20 In case the analyte is a nucleic acid, these nucleic acids preferably are naturally occurring DNA or RNA oligonucleotides.

In one embodiment the present invention relates to a method for measuring an analyte by an in vitro method, the method comprising the steps of (a) providing a sample suspected or known to comprise the analyte, (b) contacting said sample with a conjugate according between an affinity binding agent and a compound
25 according to Formula I as disclosed in the present invention under conditions appropriate for formation of an analyte conjugate complex, (c) measuring the complex formed in step (b) and thereby obtaining a measure of the analyte.

30 In one embodiment the measurement in the above method for detection of an analyte is performed by using an electrochemiluminescence based detection procedure. Also preferred the method is practiced in an aqueous solution.

The following examples are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is

understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Example 1

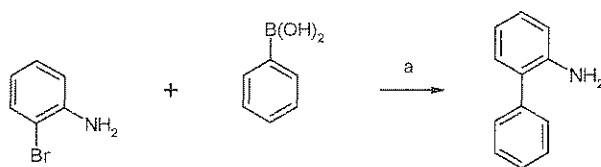
Synthesis of substituted phenyl-phenanthridines

5 **Example 1.1**

General procedure for the synthesis of substituted 2-aminobiphenyls:

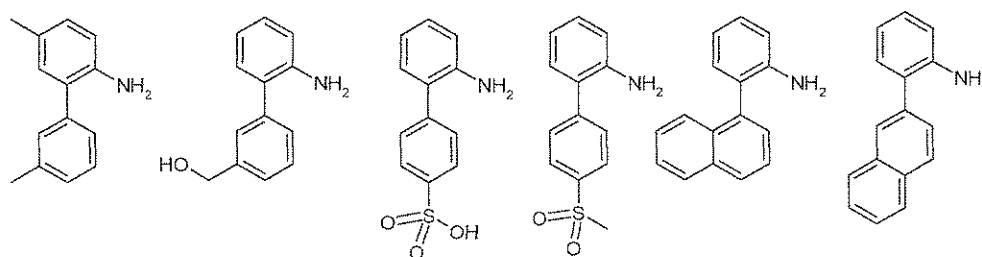
With the Suzuki-Miyaura coupling reaction as described by Youn, S.W., in Tetrahedron Lett. 50 (2009) 4598-4601 between commercially available 2-bromoaniline derivatives and the corresponding arylboronic acid the appropriate 2-aminobiphenyls can be synthesized, which are required for further reactions to phenanthridines.

Typical procedure:



a: 10 mol % $\text{PdCl}_2(\text{PPh}_3)_2$, K_2CO_3 , DMF/ H_2O (5/1), 80 °C, 24 h

15 **Other Examples:**



Example 1.2

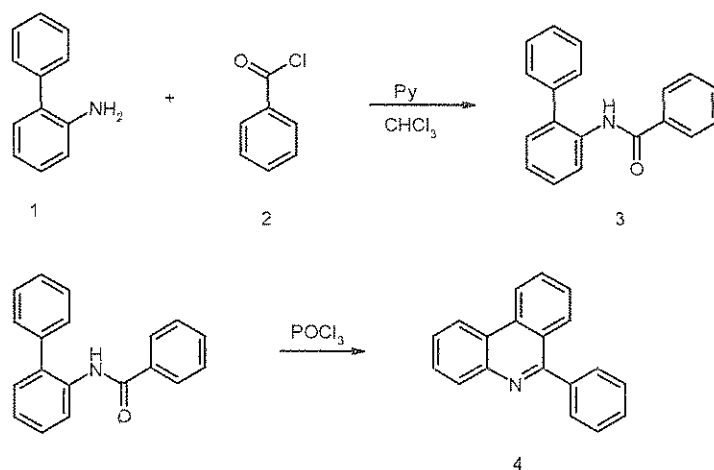
General procedure for the synthesis of substituted phenanthridines:

To the ice-cooled solution of 2-arylaniline **1** (0.01 mol) in chloroform (20 ml) was added aryl acid chloride **2** (0.01 mol) and stirred under inert condition for 30 min at room temperature. The resulting mixture was refluxed with stirring for the next 2 hours. The reaction mixture was treated by the dropwise addition of pyridine (0.02

- 18 -

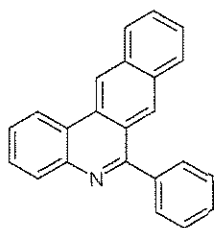
mol in 10 ml chloroform) over a period of 60 minutes. The mixture was allowed to cool to room temperature and stirred overnight. The mixture was washed well with 0.5 M HCl, dried over MgSO_4 and concentrated in vacuum. The crude product was purified by flash chromatography on silica gel, 3:2 hexane/ethyl acetate to give pure product **3** in 66% yield.

Benzamido-2-biphenyl **3** (0.01 mol) and POCl_3 (5 ml) in 20 ml of toluene were refluxed and stirred under nitrogen for 18 hours, following the procedure described by Lion, C., in Bull. Soc. Chim. Belg. 98 (1989) 557-566. The cooled reaction mixture was diluted with CH_2Cl_2 (30 ml) and poured into ice, washed with 25% NH_4OH and distilled water. The organic layer was dried over MgSO_4 and concentrated in vacuo, followed by flash chromatography (silica gel, 1:1 hexane/ethyl acetate) gave the product **4**, 6-phenylphenanthridine.



Yield: 52%. White solid. ^1H NMR (CDCl_3 , 400 MHz) δ 7.54-7.85 (m, 9H), 8.10 (d, $J = 8.0$ Hz, 1H), 8.28 (d, $J = 7.9$ Hz, 1H), 8.62 (d, $J = 8.4$ Hz, 1H), 8.67 (d, $J = 8.4$ Hz, 1H).

Using 2-naphthalen-2-yl-phenylamine instead of 2-aryl-aniline:



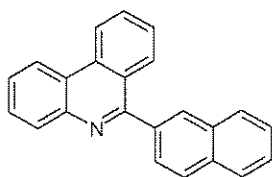
^1H -NMR (400 MHz, CDCl_3) δ 8.64 (d, $J = 9.1$ Hz, 2H), 8.29 (d, $J = 8.1$ Hz, 1H), 8.16 (d, $J = 8.92$ Hz, 1H), 7.92 (d, $J = 7.48$ Hz, 1H), 7.79-7.75 (m, 2H), 7.69 (t, $J =$

- 19 -

14.0, 8.2 Hz, 1H), 7.63-7.61 (m, 2H), 7.53-7.46 (m, 4H), 7.19 (t, J = 14.3, 7.2 Hz, 1H).

MS: $[M+H]^+$ 306.3

Using naphthalene-carbonyl chloride instead of phenyl acid chloride:



$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.74 (d, J = 8.3 Hz, 1H), 8.65 (d, J = 8.1 Hz, 1H), 8.27 (d, J = 8.1 Hz, 1H), 8.23 (s, 1H), 8.15 (d, J = 8.3 Hz, 1H), 8.03 (d, J = 8.4 Hz, 1H), 7.97-7.94 (m, 2H), 7.90-7.85 (m, 2H), 7.80-7.69 (m, 2H), 7.62 (t, J = 14.2, 7.1 Hz, 1H), 7.59-7.55 (m, 2H).

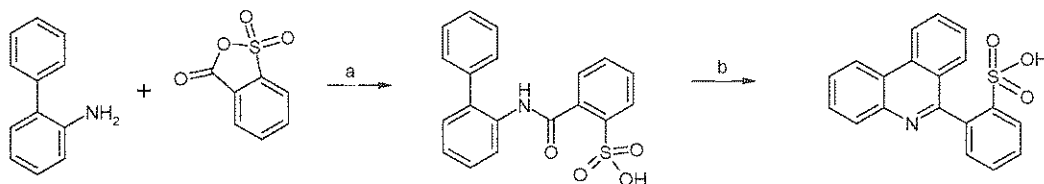
MS: $[M+H]^+$ 306.3

Example 1.3

Procedure for the synthesis of 6-(2-sulfophenyl) phenanthridine

The 6-(2-sulfophenyl)phenanthridine can be synthesized by gentle heating of arylaniline (0.01 mol) with 2-sulfobenzoic acid cyclic anhydride (0.01 mol) in CH_3CN for 6 hours using the procedure as described by Nicolai, E., in Chem. Pharm. Bull. 42 (1994) 1617-1630.

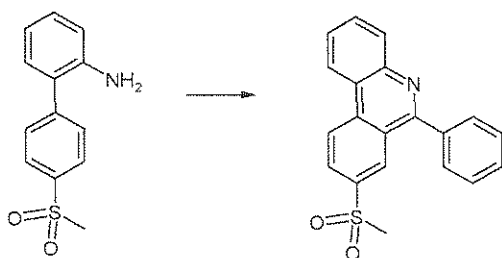
After purification the product can be converted to the appropriate phenanthridine based on the method described in example 1.2.



Example 1.4**Procedure for the synthesis of 6-phenyl-alkylsulfonyl phenanthridine**

5 The 6-phenyl-alkylsulfonyl phenanthridine can be synthesized by gentle heating of alkylsulfonyl-arylaniline (0.01 mol) with benzoic acid chloride (0.01 mol) in chloroform using the procedure as described by Lion, C., in Bull. Soc. Chim. Belg. 98 (1989) 557-566, see example 1.2.

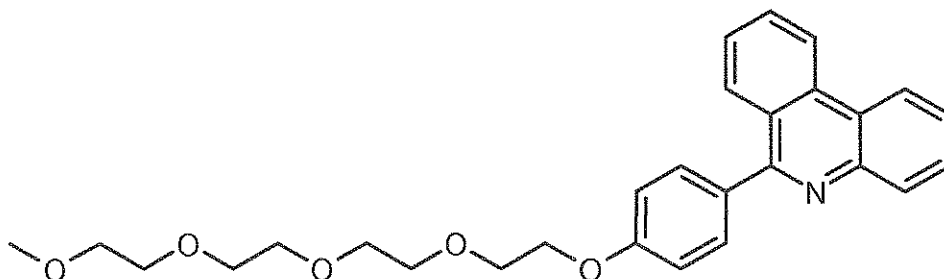
After purification the product can be converted to the appropriate phenanthridine based on the method described in example 1.2.



10 ¹H-NMR (400 MHz, CDCl₃) δ 8.92 (d, J = 8.7 Hz, 1H), 8.75 (d, J = 1.9 Hz, 1H), 8.68 (d, J = 7.0 Hz, 1H), 8.35 (dd, J = 8.7, 2.0 Hz, 1H), 8.30 (d, J = 7.2 Hz, 1H), 7.89 (t, J = 15.3, 7.1 Hz, 1H), 7.81-7.73 (m, 3H), 7.64-7.56 (m, 3H) 3.12 (s, 3H).

MS: [M+H]⁺ 334,3

15 The 6-(4-methylsulfonylphenyl)phenanthridine can be also prepared by following the procedure described by Cymerman, J., in J. Chem. Soc. (1949) 703-707.

Example 1.5**Synthesis of 6-[4-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-phenyl]-phenanthridine****5 Synthesis of 2,5,8,11-tetraoxatridecan-13-ol tosylate:**

Procedure: (JACS, 2007, 129, 13364) To a solution of 2,5,8,11-tetraoxatridecan-13-ol (7 g, 33.6 mmol) and triethylamine (4.9 ml, 35.3 mmol) in dry CH₂Cl₂ (100 ml), 4-toluenesulfonyl chloride (6.7 g, 35.3 mmol) and DMAP (120 mg) were added. The mixture was stirred at room temperature for 20 h. The reaction mixture was washed with 80 mL of HCl (1M) and then water. The extract was dried over anhydrous MgSO₄, filtrated, and the filtrate was evaporated. The residue was used in the next step without further purification.

Yield: 11.0 g (90%)

NMR:

¹H NMR (400 MHz, CDCl₃) δ 7.75 – 7.64 (m, 2H), 7.31 – 7.26 (m, 2H), 4.16 – 4.06 (m, 2H), 3.62 (m 2H), 3.59 – 3.40 (m, 10H), 3.30 (s, 3H), 2.38 (s, 3H).

¹³C{¹H} NMR (101 MHz, CDCl₃) δ 144.75 (s), 132.90 (s), 129.77 (s), 127.8 (s), 71.82 (s), 70.60 (s), 70.48 (s), 70.47 (s), 70.41 (s), 70.39 (s), 69.23 (s), 68.55 (s), 58.90 (s), 21.53 (s).

20 Synthesis of 4-PEG4-benzoic acid ethyl ester:

Procedure: (JACS, 2007, 129, 13364) A mixture of compound ethyl 2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate (8.1 g, 22.3 mmol), 4-hydroxybenzoic acid ethyl ester (3.7 g, 22.3 mmol), K₂CO₃ (15.4 g, 111.5 mmol) and 18-crown-6 (0.59 g, 2.2 mmol) was refluxed in acetone (120 ml) for 22 h. The reaction mixture was concentrated and extracted with ethyl acetate. The extract was washed with H₂O, dried over anhydrous MgSO₄, and filtrated. The filtrate was

- 22 -

evaporated to dryness and the residue was purified by column chromatography on silica gel (dichloromethane/methanol = 100:1) to obtain the compound (1.93 g, 88%).

Yield: 7 g (88%)

5 **NMR:**

^1H NMR (400 MHz, CDCl_3) δ 8.01 – 7.84 (m, 2H), 6.96 – 6.85 (m, 2H), 4.29 (q, J = 7.1 Hz, 2H), 4.12 (dd, J = 5.4, 4.3 Hz, 2H), 3.82 (dd, J = 5.4, 4.2 Hz, 2H), 3.71 – 3.56 (m, 10H), 3.51 – 3.45 (m, 2H), 3.32 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H).

10 $^{13}\text{C}\{^1\text{H}\}$ NMR (101 MHz, CDCl_3) δ 166.29 (s), 162.47 (s), 131.45 (s), 123.01 (s), 114.11 (s), 71.90 (s), 70.84 (s), 70.60 (s), 70.59 (s), 70.58 (s), 70.48 (s), 69.51 (s), 67.54 (s), 60.57 (s), 58.98 (s), 14.35 (s).

MS(+):

15 $[\text{M}+\text{Na}]^+ = \text{calc. } 379.1727, \text{ found } 379.1743$

Synthesis of 4-PEG4-benzoic acid:

Procedure: (JACS, 2007, 129, 13364) A mixture of compound ethyl 4-(2,5,8,11-tetraoxatridecan-13-yloxy)benzoate (7 g, 19.6 mmol), and KOH (2.3 g, 41.24 mmol) in 200 mL of EtOH/H₂O (1:1 v/v) was reflux overnight. After cooling down, the mixture was neutralized with HCl (2N). The resulting mixture was extracted with EtOAc and evaporated to dryness. The resulting white solid was recrystallized in EtOAc/hexanes.

Yield: 5.3 g (85%)

NMR:

25 ^1H NMR (300 MHz, CDCl_3) δ 11.17 (s, 1H), 8.14 – 7.89 (m, 2H), 7.03 – 6.75 (m, 2H), 4.29 – 4.02 (m, 2H), 3.92 – 3.81 (m, 2H), 3.78 – 3.57 (m, 10H), 3.57 – 3.46 (m, 2H), 3.35 (s, 3H).

30 $^{13}\text{C}\{^1\text{H}\}$ NMR (75 MHz, CDCl_3) δ 171.46 (s), 163.24 (s), 132.30 (s), 121.98 (s), 114.33 (s), 71.96 (s), 70.91 (s), 70.67 (s), 70.66 (s), 70.64 (s), 70.54 (s), 69.55 (s), 67.66 (s), 59.08 (s).

MS(-):

$[\text{M}-\text{H}]^- = \text{calc. } 327.1438, \text{ found } 327.1456$

Synthesis of N-Biphenyl-2-yl-4-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-benzamide:

Procedure: To a solution of 4-(2,5,8,11-tetraoxatridecan-13-yloxy)benzoic acid (3 g, 9.14 mmol), 0.2 mL of DMF in 30 mL dry DCM at 0°C, oxalyl chloride (1.05 mL, 12.34 mmol) was added. The reaction mixture was stirred at 0°C for 1 h. The solution was concentrated to dryness. The oily residue was used without further purification in the next step.

A solution of 2-phenylaniline (1.6 g), pyridine (2.4 mL) in chloroform (80 mL) under inert atmosphere was cooled down to 0 °C. (phenyl-4-(2,5,8,11-tetraoxatridecan-13-yloxy)benzoyl chloride (3.1 g, 9.14 mmol) in 20 mL was slowly added to the solution and the final mixture allowed to reach room temperature. The solution was reflux for 2 h and stirred overnight at room temperature. The reaction mixture was extracted with HCl (1 M, 2 x 100 mL), NaHCO₃ (100 mL) and water (50 mL). The organic phase was dried with MgSO₄ and purified by chromatography in silica gel (EtOAc/hexane).

Yield: 4.1 (90%)

NMR:

¹H NMR (400 MHz, CDCl₃) δ 8.49 (dd, *J* = 8.3, 0.9 Hz, 1H), 7.94 (s, 1H), 7.61 – 7.35 (m, 9H), 7.33 – 7.25 (m, 1H), 7.19 (m, 1H), 6.91 – 6.84 (m, 2H), 4.16 – 4.10 (m, 2H), 3.85 (m, 2H), 3.77 – 3.58 (m, 10H), 3.56 – 3.49 (m, 2H), 3.36 (s, 3H).

¹³C{¹H} NMR (101 MHz, CDCl₃) δ 164.56 (s), 161.65 (s), 138.18 (s), 135.12 (s), 132.32 (s), 129.97 (s), 129.39 (s), 129.22 (s), 128.66 (s), 128.57 (s), 128.16 (s), 127.13 (s), 124.18 (s), 121.23 (s), 114.57 (s), 71.95 (s), 70.89 (s), 70.64 (s), 70.63 (s), 70.54 (s), 69.54 (s), 67.63 (s), 59.04 (s), 53.51 (s).

MS(+)

[M+H]⁺ = calc. 480.2386 found. 480.2383; [M+Na]⁺ = calc. 502.2200, found 502.2204

Synthesis of 6-[4-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-phenyl]-phenanthridine:

Procedure: N-Biphenyl-2-yl-4-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-benzamide (4 g, 8.34 mmol), POCl₃ (10 ml) in 10 ml toluene were refluxed for 20 h. The mixture was cooled down to room temperature, and 100 ml

- 24 -

of dichloromethane were added. The solution was poured into ice and the mixture neutralized with NH₄OH (20%). The organic phase was extracted and washed successively with distilled water and brine, and dried over MgSO₄. The resulting solution was purified by flash chromatography (silica gel, in ethyl acetate/hexane 1:1, R_f = 0.14).

Yield: 1 g (25%)

NMR:

¹H NMR (300 MHz, CDCl₃) δ 8.68 (d, *J* = 8.3 Hz, 1H), 8.59 (dd, *J* = 8.1, 1.4 Hz, 1H), 8.23 (dd, *J* = 8.1, 1.1 Hz, 1H), 8.15 (dd, *J* = 8.3, 0.7 Hz, 1H), 7.84 (ddd, *J* = 8.3, 7.1, 1.3 Hz, 1H), 7.79 – 7.57 (m, 5H), 7.15 – 7.03 (m, 2H), 4.29 – 4.19 (m, 2H), 3.93–3.90 (m, 2H), 3.80 – 3.60 (m, 12H), 3.59 – 3.49 (m, 2H), 3.37 (s, 3H).

¹³C{¹H} NMR (75 MHz, CDCl₃) δ 160.92 (s), 159.45 (s), 143.84 (s), 133.59 (s), 131.26 (s), 130.61 (s), 130.26 (s), 129.05 (s), 128.90 (s), 127.19 (s), 126.85 (s), 125.39 (s), 123.70 (s), 122.29 (s), 122.01 (s), 114.68 (s), 72.02 (s), 70.97 (s), 70.74 (s), 70.72 (s), 70.69, 70.62 (s), 69.80 (s), 67.68 (s), 59.15 (s).

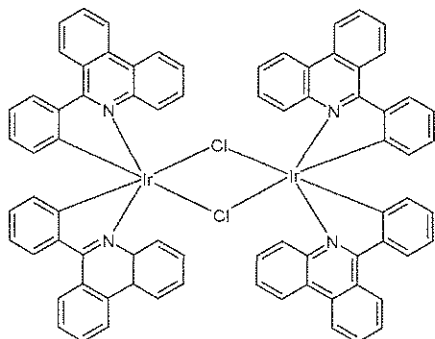
MS (+) JM358-F5, [M+H]⁺ calc = 462.2280, found 462.2275

Example 2

General procedure for the synthesis of chloro-cross-linked dimer complex:

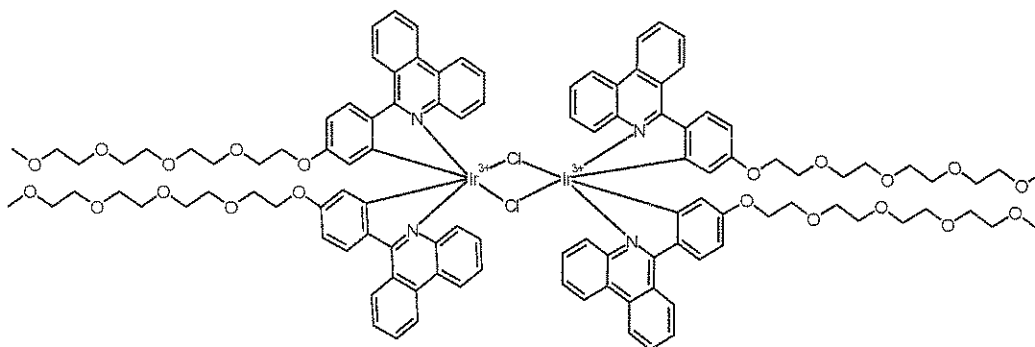
The general procedure was published by Nonoyama, M., J. Organomet. Chem. 86 (1975) 263-267.

The iridium dimers were synthesized as follow: IrCl₃•3H₂O and 2.5 equiv of 6-phenylphenanthridine were heated at 120 °C for 18 h under nitrogen in 2-ethoxyethanol/water mixture (3:1, v/v). After being cooled to room temperature the precipitate was filtered off and successively washed with methanol and Et₂O, dried to afford the desired dimer.

Example 2.1**Complex with unsubstituted phenylphenanthridine**

$[(6\text{-phenylphenanthridine})_2\text{IrCl}]_2$.

- 5 Yield: 71%. Brown solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 6.45 (d, $J = 6.8$, 4H), 6.58 (t, $J = 7.1$, 13.9 Hz, 4H), 6.95 (t, $J = 7.1$, 14.2 Hz, 4H), 7.56 (t, $J = 7.4$, 16.0 Hz, 4H), 7.68 (t, $J = 8.1$, 16.2 Hz, 4H), 7.93 (t, $J = 8.0$, 14.6 Hz, 4H), 8.07-8.13 (m, 8H), 8.80 (d, $J = 7.3$ Hz, 4H), 8.93-9.01 (m, 12H).

Example 2.2**Complex with substituted phenylphenanthridine**

- 15 A mixture of 6-[4-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-phenyl]-phenanthridine (1 g, 2.16 mmol), $\text{IrCl}_3 \cdot 3\text{H}_2\text{O}$ (346 mg, 0.98 mmol) in 16 ml of 2-EtOEtOH: H_2O (12:4) was refluxed overnight under nitrogen atmosphere. The reaction mixture was cooled down to room temperature and 60 ml of water were added to obtain an oily precipitate. The supernatant was discarded and 50 ml of water were added to the residue. The mixture was stirred for 1 h to obtain a red-brownish precipitate. The solid was filtrated and washed with water (50 ml) and
- 20 Et_2O (30 ml). The brown solid was dissolved in the smaller amount of

dichloromethane and precipitated upon addition of Et₂O. It was used in the next step without further purification.

Yield: 550 mg (50%)

NMR:

5 ¹H NMR (300 MHz, CDCl₃) δ 8.74 (d, J = 8.1 Hz, 4H), 8.36 (dd, J = 8.0, 5.2 Hz, 8H), 7.90 (dd, J = 14.7, 7.7 Hz, 8H), 7.81 (d, J = 9.0 Hz, 4H), 7.79 – 7.67 (m, 4H), 6.78 – 6.65 (m, 4H), 6.32 (dd, J = 8.8, 2.5 Hz, 4H), 5.89-5.83 (m, 4H), 5.28 (d, J = 2.5 Hz, 4H), 3.67-3.10 (m, 100H, PEG Chain, contains some impurities)

MS(ESI-MS(+)):

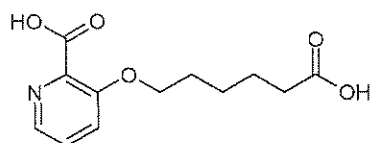
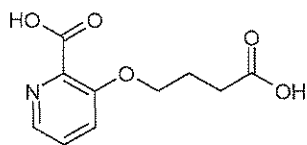
10 [M+2Na⁺]²⁺ calc. 1171.3463, found 1171.3473; [(C[^]N)₂Ir]⁺ = calc. 1113.3877, found 1113.3892

Example 3

A) Synthesis of Carboxyalkylenoxy-picolinic acid derivatives:

15 A mixture of the 3-hydroxy-2-pyridinecarboxylic acid (0.01 mol), the ethyl 4-bromobutanoate or ethyl 6-bromohexanoate (0.021 mol), and a mixture of potassium carbonate (5 eq.) in DMF (20 ml) was heated at 90 °C for 20 hours under nitrogen. After cooling, the reaction mixture was poured into ice-water mixture and extracted three times with dichloromethane (30 ml), dried over anhydrous MgSO₄, filtered, and the solvent was evaporated to dryness. Purified by flash
20 chromatography (silica, hexane/ethyl acetate 3:1) to afford the product (based on Patent US 5,219,847).

The formed ester was hydrolyzed by NaOH in MeOH (pH = 10). The pH of the solution was then adjusted to 6.0 and stirred at r.t. overnight. The solvent was removed in vacuo and the residue was crystallized from hexane/acetone to give the
25 desired product.

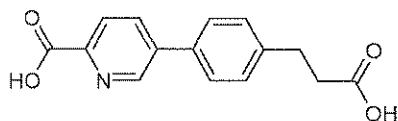


3-(Carboxy-pentyloxy)-pyridine-2-carboxylic acid. Yield: 51%. Gray solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 1.39-1.45 (m, 2H), 1.51-1.57 (m, 2H), 1.67-1.74 (m, 2H), 2.19-2.23 (m, 2H), 4.04-4.07 (m, 2H), 7.47-7.50 (m, 1H), 7.61 (d, $J = 8.1$ Hz, 1H), 8.13 (d, $J = 8.1$ Hz, 1H).

5 **B) Synthesis of 5-[4-(2-Carboxy-ethyl)-phenyl]-pyridine-2-carboxylic acid**

Under an argon atmosphere, to 4 ml 1,2-dimethoxyethane are added 5-bromo-pyridine-2-carboxylic acid (93 mg, 0.46 mmol), 4-(2-carboxyethyl)benzeneboronic acid (106 mg, 0.55 mmol), 0.51 ml of a 2M aqueous sodium carbonate solution and dichlorobis- (triphenylphosphin) palladium (II) (20 mg, 0.03 mmol). The mixture is stirred at 90 °C overnight, cooled and quenched with water. Ethyl acetate is added and the mixture adjusted to pH = 2 with 1M hydrochloric acid. After threefold extraction with ethyl acetate, the combined organic layers are dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue is purified by silica gel chromatography (eluent: dichloromethane/methanol 5: 1).

15



^1H -NMR (400 MHz, DMSO- d_6) δ 9.00 (d, $J = 2$ Hz, 1H), 8.25 (dd, $J = 8.2, 2.3$ Hz, 1H), 8.11 (d, $J = 8.1$ Hz, 1H), 7.74 (d, $J = 8.2$ Hz, 2H), 7.41 (d, $J = 8.2$ Hz, 2H), 2.91 (t, $J = 15, 7.5$ Hz, 2H), 2.61 (t, $J = 15.1, 7.6$ Hz, 2H).

20

MS: $[\text{M}+\text{H}]^+$ 272.3

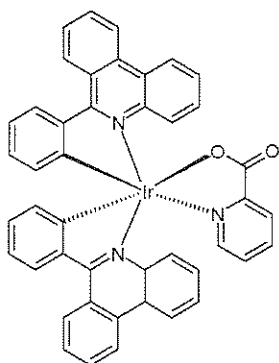
Example 4

General procedure for the synthesis of Iridium complexes

A chloro-cross-linked dimer complex 0.5 mmol, picolinate 1.25 mmol and Na_2CO_3 3 mmol were mixed into 2-ethoxyethanol (12 ml) and heated at 120 °C for 15 hours. To the cooled mixture distilled water was added (25 ml), the crude product was then filtered off and washed with water, followed by portions of n-hexane and Et_2O . The product was purified by column chromatography (silica, n-hexane/dichloromethane) to give a red powder.

30

(based on Lamansky, S., Inorg. Chem. 40 (2001) 1704-1711)



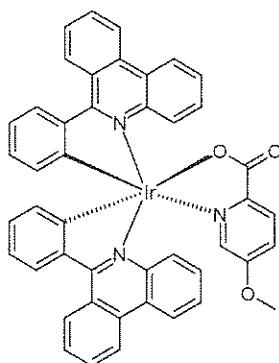
Ir(6-phenylphenanthridine)₂ Pyridine-2-carboxylic acid:

5

¹H NMR (400 MHz, CDCl₃) δ 9.17 (d, J = 7.8 Hz, 1H), 9.09 (d, J = 8.2 Hz, 1H), 8.71 (d, J = 8.2 Hz, 1H), 8.62 (t, J = 14.8, 7.8 Hz, 2H), 8.43-8.33 (m, 4H), 8.23 (d, J = 8.1 Hz, 1H), 7.92-7.77 (m, 4H), 7.65 (t, J = 15, 7.9 Hz, 2H), 7.57-7.46 (m, 3H), 7.36 (t, J = 14.8, 7.8 Hz, 1H), 7.19-7.16 (m, 2H), 7.10 (d, J = 7.8 Hz, 1H), 7.04 (t, J = 14.2, 6.8 Hz, 1H), 6.92 (t, J = 14.1, 6.7 Hz, 1H), 6.80 (t, J = 13.7, 6.8 Hz, 1H), 6.67 (t, J = 13.7, 6.6 Hz, 1H), 6.51 (d, J = 6.8 Hz, 1H).

10

MS: [M+H]⁺ 826.4



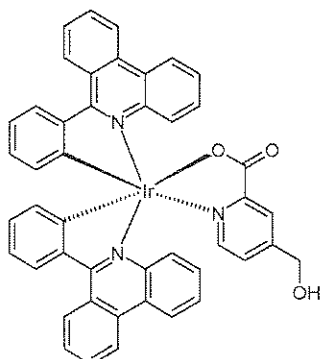
15

Ir(6-phenylphenanthridine)₂ 5-(Methoxy)pyridine-2-carboxylic acid:

¹H-NMR (400 MHz, CDCl₃) δ 9.15 (d, J = 8.2 Hz, 1H), 9.09 (d, J = 8.2 Hz, 1H), 8.70 (d, J = 7.8 Hz, 1H), 8.61 (d, J = 8.2 Hz, 2H), 8.44-8.35 (m, 3H), 8.21 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 2.7, 1H), 7.91-7.86 (m, 2H), 7.82-7.80 (m, 2H), 7.68 (d, J = 8.6 Hz, 1H), 7.57-7.53 (m, 3H), 7.36 (t, J = 15.2, 7.2 Hz, 1H), 7.14 (t, J = 15.1, 7.6 Hz, 1H), 7.08-6.93 (m, 4H), 6.78 (t, J = 14.9, 7.6 Hz, 1H), 6.65 (t, J = 14.8, 7.6 Hz, 1H), 6.49 (d, J = 7.6 Hz, 1H), 3.63 (s, 3H).

20

MS: $[M+H]^+$ 854.2



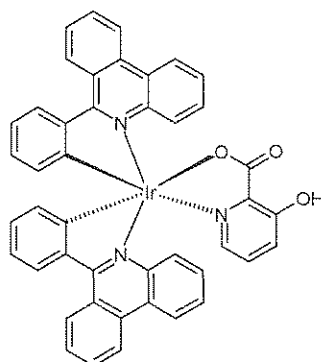
5

Ir(6-phenylphenanthridine)₂ 4-(Hydroxymethyl)pyridine-2-carboxylic acid:

¹H-NMR (400 MHz, DMSO-d₆) δ 9.14 (d, J = 8.1 Hz, 2H), 8.96 (d, J = 8.0 Hz, 1H), 8.87 (d, J = 7.7 Hz, 1H), 8.73 (d, J = 7.7 Hz, 1H), 8.68 (d, J = 7.8 Hz, 1H), 8.51 (d, J = 8.6 Hz, 1H), 8.37 (d, J = 8.2 Hz, 1H), 8.26-8.24 (m, 2H), 8.10 (t, J = 14.7, 7.3 Hz, 1H), 8.02-7.96 (m, 3H), 7.68 (d, J = 8.4 Hz, 1H), 7.62 (t, J = 15.2, 7.1 Hz, 1H), 7.53-7.48 (m, 2H), 7.39-7.37 (m, 2H), 7.16 (t, J = 15.3, 7.2 Hz, 1H), 7.10-7.04 (m, 2H), 6.86 (d, J = 6.8 Hz, 1H), 6.78 (t, J = 14.2, 7.1 Hz, 1H), 6.67 (t, J = 14.9, 7.3 Hz, 1H), 6.35 (d, J = 6.8 Hz, 1H), 5.32 (s, 1H), 4.33 (s, 2H).

15

MS: $[M+H]^+$ 854.2



20

Ir(6-phenylphenanthridine)₂ 3-Hydroxypyridine-2-carboxylic acid:

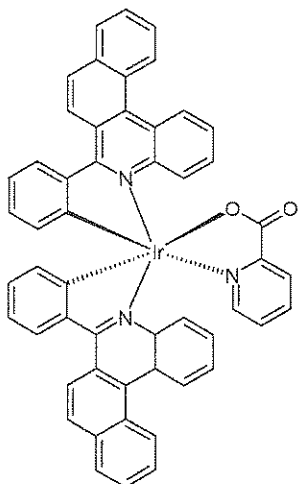
¹H-NMR (400 MHz, CDCl₃) δ 9.15 (d, J = 8.3 Hz, 1H), 9.06 (d, J = 8.2 Hz, 1H), 8.65-8.57 (m, 3H), 8.46-8.41 (m, 2H), 8.34 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 8.0 Hz, 1H), 7.94-7.78 (m, 5H), 7.72 (d, J = 7.8 Hz, 1H), 7.58-7.55 (m, 2H), 7.40 (t, J =

- 30 -

14.0, 7.0 Hz, 1H), 7.15 (t, $J = 15.2$, 7.0 Hz, 1H), 7.05-6.95 (m, 5H), 6.77 (t, $J = 13.7$, 7.0 Hz, 1H), 6.66 (t, $J = 13.6$, 6.4 Hz, 1H), 6.50 (d, $J = 6.6$ Hz, 1H).

MS: $[M+H]^+$ 839.2

5

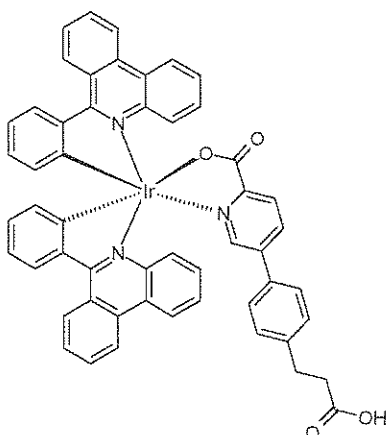


Ir(6-phenyl-benzophenanthridine)₂ Pyridine-2-carboxylic acid:

10 ¹H NMR (400 MHz, CDCl₃) δ 9.04 (m, 4H), 8.82 (m, 2H), 8.77-8.70 (m, 1H), 8.41 (d, $J = 8.0$ Hz, 1H), 8.29-8.27 (m, 2H), 8.15-8.09 (m, 4H), 7.85 (d, $J = 8.3$ Hz, 1H), 7.78-7.71 (m, 4H), 7.65 (d, $J = 7.7$ Hz, 1H), 7.62-7.553 (m, 2H), 7.45-7.40 (m, 2H), 7.23-7.17 (m, 1H), 7.13-7.05 (m, 3H), 7.05-7.00 (m, 1H), 6.83 (dd, $J = 10.8$, 4.0 Hz, 1H), 6.68 (dd, $J = 10.9$, 3.8 Hz, 1H), 6.51 (dd, $J = 7.6$, 0.9 Hz, 1H).

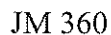
15

MS: $[M+H]^+$ 924.2



20 Ir(6-phenylphenanthridine)₂ 2-(Carboxyethyl-phenyl)pyridine-2-carboxylic acid:

Synthesis of JM 360:



Yield: 30%.

25 ¹H NMR (300 MHz, CDCl₃) δ 9.06 (d, *J* = 8.2 Hz, 1H), 8.98 (d, *J* = 8.1 Hz, 1H), 8.61 (m, 3H), 8.46 – 8.21 (m, 4H), 8.13 (d, *J* = 8.9 Hz, 1H), 7.83 (m, 4H), 7.61 (m, 2H), 7.57 – 7.41 (m, 3H), 7.30 (d, *J* = 7.2 Hz, 1H), 7.24 – 7.12 (m, 1H), 6.89 (t, *J* =

7.2 Hz, 1H), 6.76 (dd, $J = 8.9, 2.5$ Hz, 1H), 6.61 (dd, $J = 8.8, 2.6$ Hz, 1H), 6.54 (d, $J = 2.5$ Hz, 1H), 5.99 (d, $J = 2.6$ Hz, 1H), 3.85 – 3.41 (m, 32H), 3.34 (s, 3H), 3.33 (s, 3H).

MS: $[2M+2Na]^{2+}$ calc. 1258.4012, found 1258.4030. $[M+H]^+$ calc. 1236.4197, found 1236.4227

Example 5

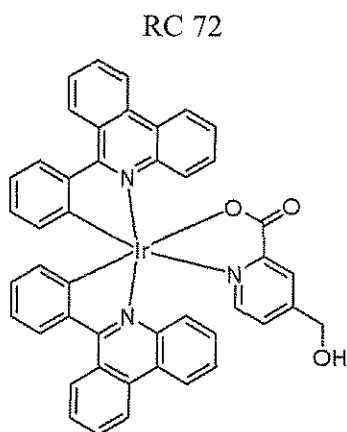
ECL with a novel Iridium complex

The electrochemiluminescence signal of several metal complexes was assessed in an ELECSYS[®] analyzer (Roche Diagnostics GmbH). Measurements were carried out homogeneously in the absence of streptavidin-coated paramagnetic microparticles. Stock solutions of each metal complex at 0.1 mg/ml DMSO were diluted with PBS buffer resulting in 10 nM solutions. The 10 nM solutions were handled as samples on the ELECSYS[®] analyzer. 20 μ l sample was incubated together with 90 μ l Reagent 1 (ProCell) and 90 μ l Reagent 2 (ProCell) for 9 min at 37°C and subsequently the electrochemiluminescence signal was quantified.

ECL results:

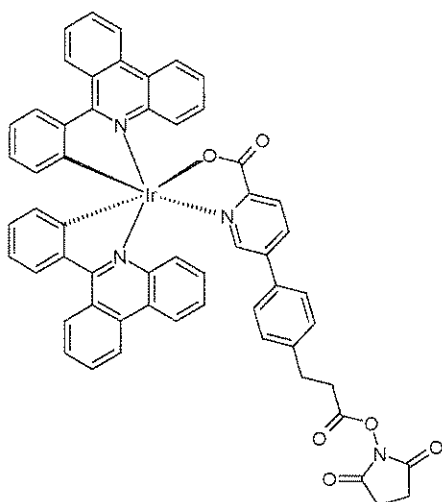
Reference Ru(bpy)₃ = 10000 counts in 10 nmolar concentration

- JM 360 = 31258 counts 10 nmolar concentration
- RC 72 = 45512 count in 10 nmolar concentration



Example 6**Synthesis of an Iridium complex with reactive group for bioconjugation**

Ir(6-phenylphenanthridine)₂ 2-(Carboxyethyl-phenyl)pyridine-2-carboxylic acid (15 mg) was dissolved in a mixture of dry acetonitrile 5mL and dry pyridine 0.01 mL. Disuccinimidyl carbonate (DSC) (1.5 eq) was added and the mixture was stirred under nitrogen at room temperature overnight. The solution was added to chloroform (10 mL), washed with 0.5 M HCl (1 x 2 mL), saturated aqueous NaHCO₃ (1 x 2 mL) and water (2 x 5 mL) dried over MgSO₄, and concentrated in vacuo to yield a red powder.



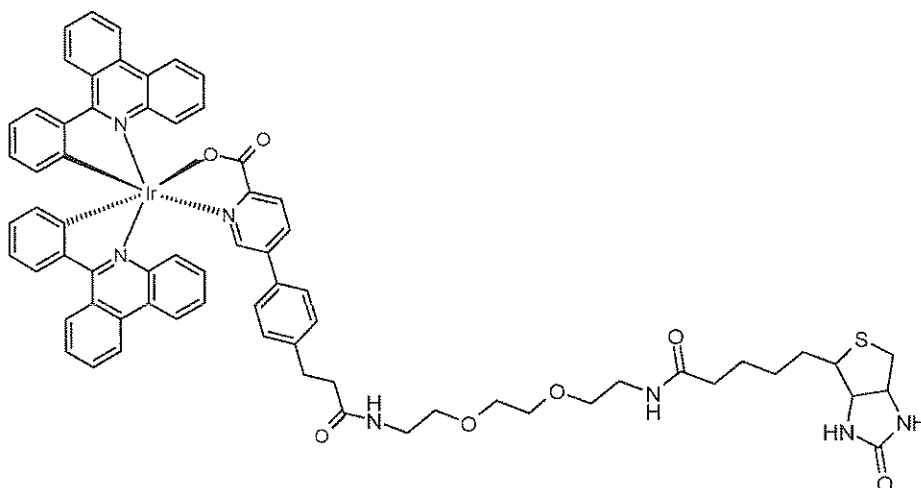
Ir(6-phenylphenanthridine)₂ 2-(Carboxyethyl-phenyl)pyridine-2-carboxylic acid N-succinimidyl ester:

¹H-NMR (400 MHz, CD₃CN) δ 9.25 (m, 1H), 9.17 (d, J = 8.0 Hz, 1H), 8.83 (d, J = 8.4 Hz, 1H), 8.75-8.68 (m, 1H), 8.60-8.54 (m, 3H), 8.47 (d, J = 8.1 Hz, 1H), 8.43 (d, J = 2.1 Hz, 1H), 8.30 (d, J = 8.1 Hz, 1H), 8.06 (t, J = 15.4, 7.2 Hz, 1H), 7.97-7.95 (m, 3H), 7.77-7.70 (m, 2H), 7.61 (t, J = 15.2, 7.0 Hz, 1H), 7.52-7.44 (m, 3H), 7.36 (d, J = 8.3 Hz, 2H), 7.18 (t, J = 15.2, 7.0 Hz, 1H), 7.12-7.09 (m, 3H), 7.04-6.98 (m, 2H), 6.78 (t, J = 14.9, 7.2 Hz, 1H), 6.71 (t, J = 14.8, 7.5 Hz, 1H), 6.57 (d, J = 7.6 Hz, 1H), 3.07-3.01 (m, 4H), 2.80 (s, 4H).

MS: [M+H]⁺ 1069.3

Example 7**Synthesis of an Iridium-complex conjugate with biotin**

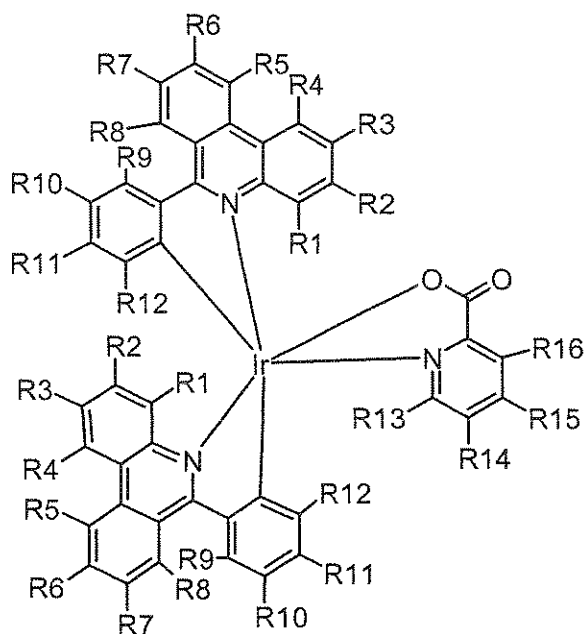
Ir(6-phenylphenanthridine)₂ 2-(Carboxyethyl-phenyl)pyridine-2-carboxylic acid
NHS ester (12 mg) and 4mg of N-Biotinyl-3,6-dioxaoctane-1,8-diamine
trifluoroacetate was dissolved in a dry DMF 5mL. Pyridine (0.016 mL in 2mL
DMF) was added and the mixture was stirred under nitrogen at room temperature
overnight. The solution was added to chloroform (10 mL), washed with 0.5 M HCl
(1 x 2 mL), saturated aqueous NaHCO₃ (1 x 2 mL) and water (2 x 5 mL) dried over
MgSO₄, and concentrated in vacuo to yield a red powder. The product was purified
by column chromatography (silica, n-hexane/ethyl acetate) to give red powder.



MS: [M+H]⁺ 1328.6

Patent Claims

1. An iridium-based chemiluminescent compound of Formula I



wherein R1-R16 is hydrogen, halide, cyano- or nitro-group, amino, alkylamino, substituted alkylamino, arylamino, substituted arylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfinio, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or R17, wherein R17 is aryl, substituted aryl, alkyl, substituted alkyl branched alkyl, substituted branched alkyl, arylalkyl, substituted arylalkyl, alkylaryl, substituted alkylaryl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl,

arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or,

5 wherein within R1-R12 or/and within R13-R16, respectively, two adjacent Rs can form an aromatic ring or a substituted aromatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate or,

15 wherein within R1-R12 or/and within R13-R16, respectively, two adjacent Rs can form an aliphatic ring or a substituted aliphatic ring, wherein the substituent is selected from hydrogen, halide, cyano- or nitro-group, a hydrophilic group, like amino, alkylamino, substituted alkylamino, alkylammonium, substituted alkylammonium, carboxy, carboxylic acid ester, carbamoyl, hydroxy, substituted or unsubstituted alkyloxy, substituted or unsubstituted aryloxy, sulfanyl, alkylsulfonyl, arylsulfonyl, sulfo, sulfino, sulfeno, sulfonamide, sulfoxide, sulfodioxide, phosphonate, phosphinate and

20 wherein at least one of R13-R16 is -Q-Y, wherein Q represents a linker and Y is a functional group.

2. The compound according to claim 1, wherein the linker Q is a straight or branched saturated, unsaturated, unsubstituted or substituted C1-C20 alkyl chain, or a 1 to 20 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S.
- 25 3. The compound according to claim 1, wherein the linker Q is a saturated C1-C12 alkyl chain or a 1 to 12 atom chain with a backbone consisting of carbon atoms and one or more heteroatoms selected from O, N and S.
4. The compound according to claim 1 or 2, wherein the functional group Y is selected from the group consisting of carboxylic acid, N-hydroxysuccinimide ester, amino group, halogen, sulfhydryl, maleimido, alkynyl, azide and phosphoramidite.
- 30

5. A conjugate comprising a compound according to any of claims 1 to 4 and covalently bound thereto an affinity binding agent.
6. The conjugate of claim 5, wherein the affinity binding agent is selected from the group consisting of antigen and antibody, biotin or biotin analogue and avidin or streptavidin, sugar and lectin, nucleic acid or nucleic acid analogue and complementary nucleic acid and receptor and ligand.
7. The conjugate according to claim 5 or 6, wherein said affinity binding agent is a nucleic acid or an antibody.
8. Use of a compound according to any of claims 1 to 4 or of a conjugate according to any of claims 5 to 7 for performing an electrochemiluminescence reaction in an aqueous solution.
9. Use of a compound according to any of claims 1 to 4 or of a conjugate according to any of claims 5 to 7 in an electrochemiluminescence based detection method.
10. Use of a compound according to any of claims 1 to 4 or of a conjugate according to any of claims 5 to 7 in the detection of an analyte.
11. A method for measuring an analyte by an in vitro method, the method comprising the steps of
 - a) providing a sample suspected or known to comprise the analyte
 - b) contacting said sample with a conjugate according to any of claims 5 to 7 under conditions appropriate for formation of an analyte conjugate complex,
 - c) measuring the complex formed in step (b) and thereby obtaining a measure of the analyte.