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(54) Title: CHEMICAL COMPOUND ARRAYS USEFUL FOR DETECTING BACTERIAL PEPTIDES

(57) Abstract: The present invention relates to chemical compound libraries and microarrays and nanoarrays comprising the chemical compound libraries. The invention also relates to assays for detecting bacterial peptides or proteins comprising contact-
ing the chemical compound array with a test sample that may contain the bacterial peptides or proteins.



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CHEMICAL COMPOUND ARRAYS USEFUL FOR DETECTING BACTERIAL PEPTIDES**Field of the Invention**

The present invention relates to chemical compound libraries and microarrays and
5 nanoarrays comprising the chemical compound libraries. The invention also relates to
assays for detecting bacterial peptides or proteins comprising contacting the chemical
compound array with a test sample that may contain the bacterial peptides or proteins.

Background of the Invention

10 Chemical compound microarrays have been made by spotting a collection of organic
chemical compounds onto a solid surface, such as glass or plastic. In terms of design and
manufacture, this microarray format is thus very similar to that employed with DNA
microarray or protein/antibody microarrays. Such chemical microarrays have
predominately found application in chemical genetics research, where advantage is taken
15 of the affinity interactions of specific chemical compounds with proteins and in general
drug discovery research, where they have found use in the search for potential drugs to
therapeutic targets. With chemical microarrays, the chemical compounds are usually
directly added to the glass or plastic surface by adding small droplets of the compounds in
different solvents or in some cases may be immobilised by covalent interaction with a
20 functionalised solid support.

However, compared to DNA or protein/antibody microarrays, the development and
application of chemical microarrays (also known as chemical compound microarrays or
small molecule microarrays) still remains challenging. These constraints have their origin
25 partly because (a) chemical microarrays can potentially be based on the use of large
numbers of chemical structures (theoretically in excess of 10^{40} compounds if the molecular
diversity of accessible organic compounds is taken into account), (b) a general
methodology is lacking to covalently link or bind this diverse variety of compounds to the
same chip surface; (c) significant differences in the solubility and reaction kinetics of the
30 compounds occur, and (d) there is an absence of general, robust and reliable methods to

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validate and quantify the chemical compounds once immobilised, in terms of concentration, density (pmole/m²) and structural stability.

To avoid constraints in covalent chemical immobilisation, alternative deposition methods
5 have been devised, which allow chemical compounds to be chemisorbed onto a slide surface, but again the unpredictable differences in dissolution rates of the compounds, and the absence of robust methods to validate and quantify the chemical compounds once chemisorbed have resulted in low levels of performance and lack of reproducibility of these types of chemical microarrays. Approaches to avoid some of these problems have
10 utilized solution-based chemical compound microarray methods, whereby the chemical compounds are individually arrayed as 'dots' or 'spots' by contact printing techniques onto a glass slide surface using a buffer that contains a low concentration of glycerol to prevent evaporation. However, the fundamental constraints remain in terms of difficulties with reproducibly and reliability at low cost, the lack of a general procedure of immobilization
15 or capture chemistry, rather than the need to use a diverse range of different attachment chemistries, difficulties in accurately determining the concentrations/densities of the chemical compounds once immobilised and the absence of procedures that enable multiple binding partners, when bound to the microarray, to be simultaneously detected and analysed, in terms of their relative abundance, composition and structure, have hitherto not
20 found practical or innovative solutions.

Chemical arrays of diverse chemical compounds can be used to detect the presence of different biological molecules in a test sample. However, these different biological molecules are usually derived from different types of organisms. In some cases it would
25 be advantageous to differentiate between very similar types of microorganisms, such as strains of a specific bacterium.

A need therefore exists for general methods of immobilization of chemical compounds for use in chemical microarray and nanoarray formats, their fabrication and their application
30 which is generic, simple to use, low cost, yet has improved precision, reproducibility and sensitivity, including the capabilities to provide quantitative assessment of the densities of

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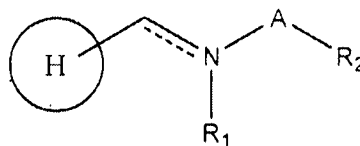
the immobilised chemical compounds, and the opportunity to provide structural information about the target molecules which bind to the so immobilised chemical compound array. There also exists a need for chemical compound libraries that can be used to identify the presence of biological molecules and in some cases, differentiate
 5 between very similar microorganisms.

Summary of the Invention

The present invention is predicated at least in part on the development of a chemical compound library which is readily immobilised to form chemical compound microarrays
 10 or nanoarrays in a generic, simple to use, low cost manner that is reproducible and sensitive. Furthermore, the chemical compound microarrays and/or nanoarrays comprising the chemical compounds in the library are suitable for detecting the presence of bacteria in a test sample and may even distinguish between particular strains of a specific bacterium.

15 Description of the Invention

In one aspect of the present invention, there is provided a chemical library comprising at least two compounds of formula (I):



(I)

wherein

H is an optionally substituted nitrogen containing heteroaryl group;

----- is selected from a single and double bond;

R₁ is hydrogen when ----- is a single bond and absent when ----- is a double bond;

25 A is a divalent linker;

R₂ is a functional group suitable for attachment to a solid support.

The compounds in the library may be suitable to be attached to the solid support in any manner that is robust enough to withstand the washing and handling conditions required

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for use of the chemical library. For example, the compounds may be suitable to be attached to the solid support by adsorption such as by ionic interactions, electrostatic interactions and hydrogen bonding. In other embodiments, the compounds may be suitable for attachment to the solid support by covalent bonding. In some embodiments, the compounds in the chemical library are suitable for attachment to the solid support by the same means such as covalent bonding. In other embodiments, at least some of the compounds in the chemical library are suitable for attachment by varying means, such as hydrogen bonding or covalent bonding. In particular embodiments, the compounds are suitable for attachment to the solid support by covalent bonding.

10

In particular embodiments of the compound of formula (I), one or more of the following applies:

H is selected from pyrrolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,3-triazinyl, indolyl, indazolyl, quinolinyl, isoquinolinyl, cinnolinyl, quinazolinyl, quinoxaliny, 1,8-naphthyridinyl, 1,7-naphthyridinyl, 1,6-naphthyridinyl, 1,5-naphthyridinyl, phthalazinyl, benzo-1,2,3-triazinyl, benzo-1,2,4-triazinyl, oxazole, thiazole, benzoxazole, benzothiazole and pteridinyl, each of which is optionally substituted; especially pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,2,3-triazinyl, 1,2,4-triazinyl, 1,3,5-triazinyl, quinolinyl and cinnolinyl, each of which is optionally substituted; more especially pyridinyl and quinolinyl, each of which is optionally substituted;

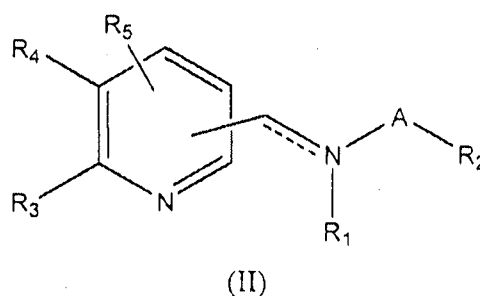
A is a divalent linker selected from $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted; especially $-(CH_2)_{1-6}-$, $-aryl-$, $-(CH_2)_{1-3}-aryl-$, $-(CH_2)_{1-3}-aryl-(CH_2)_{1-3}-$, $-aryl-(CH_2)_{1-3}-$, $-aryl-NH-(CH_2)_{1-3}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-3}-$, $-aryl-CONH-aryl-$, wherein each aryl is optionally substituted; more especially $-(CH_2)_{1-6}-$, $-phenyl-$, $-(CH_2)_{1-3}-phenyl-$, $-phenyl-(CH_2)_{1-3}-$, $-(CH_2)_{1-3}-phenyl-(CH_2)_{1-3}-$, $-phenyl-NH-(CH_2)_{1-3}-$, $-phenyl-NH-phenyl-$, $-phenyl-CONH-(CH_2)_{1-3}-$ and $-phenyl-CONH-phenyl-$, wherein each phenyl is optionally substituted; even more especially $-(CH_2)_{1-6}-$, $-phenyl-$, $-CH_2-phenyl-$, $-phenyl-CH_2-$, $-phenyl-NH-phenyl$ and $-phenyl-CONH-CH_2-$ wherein each phenyl is optionally substituted; and

30

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R₂ is -CO₂H, -NH₂ or -OH.

In one aspect of the invention there is provided a chemical library comprising at least two compounds of formula (II):



wherein

----- is a double or single bond;

R₁ is hydrogen, when ----- is a single bond and absent when ----- is a double bond;

10 A is a divalent linker;

R₂ is a functional group suitable for attachment to a solid support;

R₃ to R₅ are each independently selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl), -NH(C₁₋₆alkyl)₂, -(OCH₂CH₂)_nCH₂OH where n is 1 to 30, or R₃ and R₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R₅.

In particular embodiments of the compound of formula (II), one or more of the following applies:

20 A is a divalent linker selected from -(CH₂)₁₋₁₀-, -aryl-, -(CH₂)₁₋₅-aryl-, -(CH₂)₁₋₅-aryl-(CH₂)₁₋₅-, -aryl-(CH₂)₁₋₅-, -aryl-NH-(CH₂)₁₋₅-, -aryl-NH-aryl-, -aryl-CONH-(CH₂)₁₋₅-, -aryl-CONH-aryl-, -aryl-CO-(CH₂)₁₋₅-, and -aryl-CO-aryl-, where each aryl is optionally substituted; especially -(CH₂)₁₋₆-, -aryl-, -(CH₂)₁₋₃-aryl-, -(CH₂)₁₋₃-aryl-(CH₂)₁₋₃-, -aryl-(CH₂)₁₋₃-, -aryl-NH-(CH₂)₁₋₃-, -aryl-NH-aryl-, -aryl-CONH-(CH₂)₁₋₃-, -aryl-CONH-aryl-,
 25 wherein each aryl is optionally substituted; more especially -(CH₂)₁₋₆-, -phenyl-, -(CH₂)₁₋₃-phenyl-, -phenyl-(CH₂)₁₋₃-, -(CH₂)₁₋₃-phenyl-(CH₂)₁₋₃-, -phenyl-NH-(CH₂)₁₋₃-, -phenyl-NH-phenyl-, -phenyl-CONH-(CH₂)₁₋₃- and -phenyl-CONH-phenyl, wherein each phenyl is

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optionally substituted; even more especially $-(CH_2)_{1-6}-$, -phenyl-, -CH₂-phenyl-, -phenyl-CH₂-, -phenyl-NH-phenyl and -phenyl-CONH-CH₂- wherein each phenyl is optionally substituted;

R₂ is -CO₂H, -NH₂ or -OH,

- 5 R₃ is selected from hydrogen, -halo, -C₁₋₃alkyl, -OH, -OC₁₋₃alkyl, -NH₂, -NH(C₁₋₃alkyl) and -N(C₁₋₃alkyl)₂;

R₄ is selected from hydrogen, -halo, -C₁₋₃alkyl, -OH, -OC₁₋₃alkyl, -NH₂, -NH(C₁₋₆alkyl) and -N(C₁₋₃alkyl)₂;

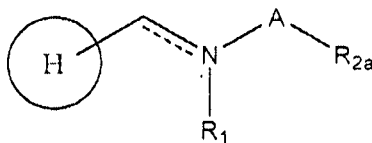
- or R₃ and R₄ taken together with the carbon atoms to which they are attached form a 6
10 membered aromatic ring optionally substituted with one or more R₅;

R₅ is selected from hydrogen, -halo, -C₁₋₃alkyl, -OH, -OC₁₋₃alkyl, -NH₂, -NH(C₁₋₃alkyl) and -N(C₁₋₃alkyl)₂; and

n is 1 to 20.

- 15 In some embodiments, the chemical library comprises more than two compounds of formulae (I) or (II), for example, at least three compounds, at least 5, 10, 15, 20 or 30 compounds of formulae (I) or (II). In some embodiments, the chemical library comprises, for example, 300 compounds or 1000 compounds.

- 20 In another aspect of the invention there is provided a chemical compound array comprising
a) a functionalised solid support; and
b) at least one compound of formula (IA):



25

(IA)

wherein

H is an optionally substituted nitrogen containing heteroaryl group;

----- is selected from a single and double bond;

R₁ is hydrogen when ----- is a single bond and absent when ----- is a double bond;

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A is a divalent linker;

R_{2a} represents a functional group attached to the functionalised solid support.

The compound may be attached to the solid support in any manner that is robust enough to withstand the washing and handling conditions required for use of the chemical array. For example, the compound may be attached to the solid support by adsorption, such as by ionic interactions, electrostatic interactions and hydrogen bonding. In other embodiments the compounds are attached to the solid support by covalent bonding. In some embodiments, the compounds in the chemical array are attached to the solid support by the same means, such as covalent bonding. In other embodiments, at least some of the compounds in the chemical array are attached by varying means, such as where some compounds are attached by hydrogen bonding and some compounds are attached by covalent bonding. In particular embodiments, the compounds are attached to the solid support by covalent bonding.

15

In particular embodiments of the compound of formula (IA), one or more of the following applies:

H is selected from pyrrolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,3-triazinyl, indolyl, indazolyl, quinolinyl, isoquinolinyl, cinnolinyl, quinazolinyl, quinoxalinyl, 1,8-naphthyridinyl, 1,7-naphthyridinyl, 1,6-naphthyridinyl, 1,5-naphthyridinyl, phthalazinyl, benzo-1,2,3-triazinyl, benzo-1,2,4-triazinyl, oxazole, thiazole, benzoxazole, benzothiazole and pteridinyl, each of which is optionally substituted; especially pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,2,3-triazinyl, 1,2,4-triazinyl, 1,3,5-triazinyl, quinolinyl and cinnolinyl, each of which is optionally substituted; more especially pyridinyl and quinolinyl, each of which is optionally substituted;

A is a divalent linker selected from $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted; especially $-(CH_2)_{1-6}-$, $-aryl-$, $-(CH_2)_{1-3}-aryl$, $-(CH_2)_{1-3}-aryl-(CH_2)_{1-3}-$, $-aryl-(CH_2)_{1-3}-$, $-aryl-NH-(CH_2)_{1-3}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-3}-$, $-aryl-CONH-aryl-$, wherein each aryl is optionally substituted; more especially $-(CH_2)_{1-6}-$, $-phenyl-$, $-(CH_2)_{1-3}-$

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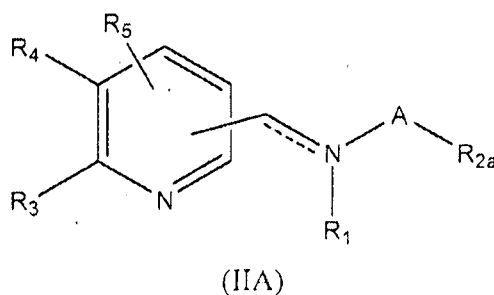
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phenyl-, -phenyl-(CH₂)₁₋₃-, -(CH₂)₁₋₃-phenyl-(CH₂)₁₋₃-, -phenyl-NH-(CH₂)₁₋₃-, -phenyl-NH-phenyl-, -phenyl-CONH-(CH₂)₁₋₃- and -phenyl-CONH-phenyl, wherein each phenyl is optionally substituted; even more especially -(CH₂)₁₋₆-, -phenyl-, -CH₂-phenyl-, -phenyl-CH₂-, -phenyl-NH-phenyl and -phenyl-CONH-CH₂- wherein each phenyl is optionally substituted; and

R_{2a} is -CO₂-solid support, -NHCO-solid support, -NHOCO-solid support, -CONH-solid support or -OCO-solid support.

In a particular embodiment, the chemical compound array comprises

- a) a functionalised solid support, and
- b) at least one compound of formula (IIA):



wherein

----- is a double or single bond;

R₁ is hydrogen, when ----- is a single bond and absent when ----- is a double bond;

A is a divalent linker;

- R_{2a} represents a functional group attached to the functionalised solid support;

R₃ to R₅ are each independently selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl), -NH(C₁₋₆alkyl)₂ and -(OCH₂CH₂)_nCH₂OH where n is 1 to 30; or R₃ and R₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted

- with one or more R₅.

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In particular embodiments of the compound of formula (IIA), one or more of the following applies:

Λ is a divalent linker selected from $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted; especially $-(CH_2)_{1-6}-$, $-aryl-$, $-(CH_2)_{1-3}-aryl$, $-(CH_2)_{1-3}-aryl-(CH_2)_{1-3}-$, $-aryl-(CH_2)_{1-3}-$, $-aryl-NH-(CH_2)_{1-3}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-3}-$, $-aryl-CONH-aryl-$, wherein each aryl is optionally substituted; more especially $-(CH_2)_{1-6}-$, $-phenyl-$, $-(CH_2)_{1-3}-phenyl-$, $-phenyl-(CH_2)_{1-3}-$, $-(CH_2)_{1-3}-phenyl-(CH_2)_{1-3}-$, $-phenyl-NH-(CH_2)_{1-3}-$, $-phenyl-NH-phenyl-$, $-phenyl-CONH-(CH_2)_{1-3}-$ and $-phenyl-CONH-phenyl$, wherein each phenyl is optionally substituted; even more especially $-(CH_2)_{1-6}-$, $-phenyl-$, $-CH_2-phenyl-$, $-phenyl-CH_2-$, $-phenyl-NH-phenyl$ and $-phenyl-CONH-CH_2-$ wherein each phenyl is optionally substituted;

R_{2a} is $-CO_2$ -solid support, $-NHCO$ -solid support, $-NHOCO$ -solid support, $-CONH$ -solid support or $-OCO$ -solid support,

R_3 is selected from hydrogen, $-halo$, $-C_{1-3}alkyl$, $-OH$, $-OC_{1-3}alkyl$, $-NH_2$, $-NH(C_{1-3}alkyl)$ and $-N(C_{1-3}alkyl)_2$;

R_4 is selected from hydrogen, $-halo$, $-C_{1-3}alkyl$, $-OH$, $-OC_{1-3}alkyl$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-N(C_{1-3}alkyl)_2$;

or R_3 and R_4 taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R_5 ;

R_5 is selected from hydrogen, $-halo$, $-C_{1-3}alkyl$, $-OH$, $-OC_{1-3}alkyl$, $-NH_2$, $-NH(C_{1-3}alkyl)$ and $-N(C_{1-3}alkyl)_2$; and

n is 1 to 20.

In some embodiments, the groups $A-R_2$ and/or $\Lambda-R_{2a}$ in the compounds of formulae I and II and/or IA and IIA is not derived from succinic acid, substituted succinic acid, succinamide or substituted succinamide.

In some embodiments, the chemical compound array comprises more than one compound of formulae (IA) or (IIA), for example, at least two compounds of formula (IA), at least 5, 10, 15, 20 or 30 compounds of formulae (IA) or (IIA). In some embodiments, the

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chemical compound array may include, for example, 300 or 1000 compounds of formulae (IA) or (IIA).

As used herein the term "nitrogen containing heteroaryl group" refers to a 5-7 membered aromatic group in which one or more carbon atoms have been replaced by nitrogen atoms. Examples of suitable nitrogen containing heteroaryl groups include, but are not limited to pyrrolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,3-triazinyl, indolyl, indazolyl, quinoliny, isoquinoliny, cinnoliny, quinazoliny, quinoxaliny, 1,8-naphthyridiny, 1,7-naphthyridiny, 1,6-naphthyridiny, 1,5-naphthyridiny, phthalazinyl, benzo-1,2,3-triazinyl, benzo-1,2,4-triazinyl, oxazole, thiazole, benzoxazole and benzothiazole and pteridinyl.

The term "divalent linker" as used herein refers to a moiety that links together the amino group and the functional group that is capable of or is attached to the solid support in the array. The divalent linker is preferably 1 to 10 atoms in length. Examples of suitable divalent linkers include, but are not limited to $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, $-aryl-CO-aryl-$, $-aryl-CO_2-(CH_2CH_2O)_{1-5}CH_2O-$, $-aryl-CONH-(CH_2CH_2O)_{1-5}CH_2O-$, $-aryl-NHCO-(CH_2CH_2O)_{1-5}CH_2O-$ and $-aryl-OC(O)CH_2CH_2O)_{1-5}CH_2O-$.

As used herein the term "functional group suitable for attachment to a solid support" refers to a functional group that in the presence of suitable reagents, undergoes reaction with a functional group on a solid support. For example, the functional group may be a group capable of forming an ester, an amide or an ether. Examples of the functional group include carboxylic acids, carboxylic anhydrides, acid chlorides, amines and hydroxy groups. In some instances, the functional group, such as a hydroxy group, can be reacted with a solid support functional group, such as an amine, using a reactive reagent such as 1,1'-carbonyldiimidazole (CDI) to form a carbamate (Bethel *et al.*, 1979, *J. Biol. Chem.*, 254, 1683-1686).

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As used herein, the term "alkyl", used either alone or in compound words, denotes saturated, straight chain or branched hydrocarbon groups typically having from 1 to 18 carbon atoms, preferably 1 to 10 or 1 to 6 or 1 to 3 carbon atoms. Examples of straight chain and branched alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, butyl, *sec*-butyl, *tert*-butyl, *n*-pentyl and branched isomers thereof, *n*-hexyl and branched isomers thereof, *n*-heptyl and branched isomers thereof, *n*-octyl and branched isomers thereof, *n*-nonyl and branched isomers thereof, and *n*-decyl and branched isomers thereof.

As used herein, the term "cycloalkyl" denotes cyclic saturated carbocyclic rings having 3 to 8 carbon atoms. Examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

The term "alkenyl" as used herein denotes groups formed from straight chain or branched hydrocarbon residues containing at least one carbon to carbon double bond including ethylenically mono-, di- or poly-unsaturated alkyl or cycloalkyl groups as previously defined, typically C₂₋₁₈ alkenyl (eg C₂₋₁₀ or C₂₋₆ or C₂₋₃). Alkenyl groups may include isolated or conjugated double bonds. Examples of alkenyl include, but are not limited to, vinyl, allyl, 1-methylvinyl, butenyl, *iso*-butenyl, 3-methyl-2-butenyl, 1-pentenyl, cyclopentenyl, 1-methyl-cyclopentenyl, 1-hexenyl, 3-hexenyl, cyclohexenyl, 1-heptenyl, 3-heptenyl, 1-octenyl, cyclooctenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-butadienyl, 1,4-pentadienyl and 1,4-hexadienyl.

As used herein, the term "aryl", used either alone or in compound words, denotes a C₆-C₁₄ aromatic hydrocarbon group. Suitable aryl groups include phenyl, biphenyl, naphthyl, tetrahydronaphthyl, anthracenyl, dihydroanthracenyl and phenanthrenyl. Preferred aryl groups include phenyl and naphthyl.

The term "heterocyclic" or "heterocyclyl" as used herein, refers to a cyclic hydrocarbon in which one to four carbon atoms have been replaced by heteroatoms independently selected from the group consisting of N, N(R), S, S(O), S(O)₂ and O. A heterocyclic ring may be

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saturated or unsaturated. Examples of suitable heterocyclyl groups include tetrahydrofuranyl, tetrahydrothiophenyl, pyrrolidinyl, pyrrolinyl, pyranyl, piperidinyl, pyrazolinyl, dithiolyl, oxathiolyl, dioxanyl, dioxinyl, morpholino and oxazinyl.

- 5 The term "heteroaryl" as used herein, represents a stable monocyclic or bicyclic ring of up to 7 atoms in each ring, wherein at least one ring is aromatic and at least one ring contains from 1 to 4 heteroatoms selected from the group consisting of O, N and S. Heteroaryl groups within the scope of this definition include, but are not limited to, acridinyl, carbazolyl, cinnolinyl, quinoxalinyl, quinazolinyl, pyrazolyl, indolyl, benzotriazolyl, furanyl, thienyl, thiophenyl, benzothienyl, benzofuranyl, benzodioxane, benzodioxin, quinolinyl, isoquinolinyl, oxazolyl, isoxazolyl, imidazolyl, pyrazinyl, pyridazinyl, pyridinyl, pyrimidinyl, pyrrolyl, tetrahydroquinoline, thiazolyl, isothiazolyl, 1,2,4-triazolyl, 1,2,4-oxadiazolyl, 1,2,4-thiadiazolyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,4,5-tetrazinyl and tetrazolyl. Particular heteroaryl groups have 5- or 6-membered rings, such as pyrazolyl, furanyl, thienyl, oxazolyl, isoxazolyl, imidazolyl, pyrazinyl, pyridazinyl, pyridinyl, pyrimidinyl, pyrrolyl, thiazolyl, isothiazolyl, 1,2,4-triazolyl and 1,2,4-oxadiazolyl and 1,2,4-thiadiazolyl.

The term "halo" as used herein refers to fluoro, chloro, bromo and iodo atoms.

20

As used herein, the term "optionally substituted" indicates that the nitrogen containing heteroaryl group or aryl group may be unsubstituted or substituted with one or more substituents. Suitable substituents include -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl), -NH(C₁₋₆alkyl)₂ or 25 -(OCH₂CH₂)_nCH₂OH where n is 1 to 30,

In preferred embodiments the chemical library comprising two or more compounds or chemical compound array includes one or more compounds selected from:

- 2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid,
30 2-(4-(pyridin-2-ylmethylanino)phenyl)acetic acid,
2-(pyridin-2-methyleneamino)benzoic acid,

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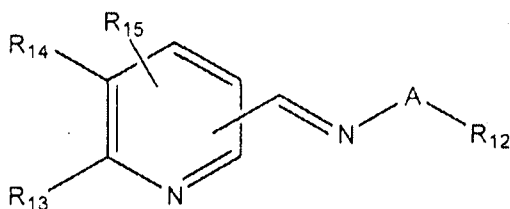
- 4-((pyridin-2-ylmethyleneamino)methyl)benzoic acid,
4-(pyridin-3-ylmethyleneamino)benzoic acid,
4-(pyridin-3-ylmethylanino)benzoic acid,
4-(pyridin-4-ylmethyleneamino)benzoic acid,
5 4-(pyridin-4-ylmethylanino)benzoic acid,
4-(pyridin-2-ylmethyleneamino)benzoic acid,
4-(pyridin-2-ylmethylanino)benzoic acid,
3-(pyridin-2-ylmethyleneamino)benzoic acid,
3-(pyridin-4-ylmethyleneamino)benzoic acid,
10 2-(4-chloro-2-(pyridin-4-ylmethyleneamino)phenylanino)benzoic acid,
2-(4-chloro-2-(pyridin-2-ylmethyleneamino)phenylanino)benzoic acid,
2-(4-(pyridin-3-ylmethyleneamino)phenyl)acetic acid,
2-(4-(pyridin-3-ylmethylanino)phenyl)acetic acid,
2-(pyridin-4-ylmethyleneamino)benzoic acid,
15 2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid,
2-(4-(pyridin-4-ylmethylanino)phenyl)acetic acid,
2-(4-(pyridin-4-ylmethyleneamino)benzamido)acetic acid,
3-(pyridin-3-ylmethyleneamino)benzoic acid,
2-(4-chloro-2-(pyridin-3-ylmethyleneamino)phenylanino)benzoic acid,
20 4-((pyridin-4-ylmethyleneamino)methyl)benzoic acid,
6-(pyridin-2-methyleneamino)hexanoic acid,
6-(pyridin-3-ylmethyleneamino)hexanoic acid,
4-(pyridin-3-ylmethylenamino)butanoic acid,
2-(4-(pyridin-3-ylmethyleneamino)benzamido)acetic acid,
25 2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylanino)benzoic acid,
3-(quinolin-2-ylmethyleneamino)benzoic acid,
2-(4-(quinolin-2-ylmethyleneamino)phenyl)acetic acid,
2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylanino)benzoic acid,
2-(2-((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylanino)benzoic acid,
30 2-(4-((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid,
3-((6-bromopyridin-2-yl)methyleneamino)benzoic acid,

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- 2-(4-((6-chloropyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(4-((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(2-((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
 2-(4-chloro-2-((6-chloropyridin-3-yl)methyleneamino)phenylamino)benzoic acid,
 5 3-((6-chloropyridin-3-yl)methyleneamino)benzoic acid,
 3-((5-bromopyridin-3-yl)methyleneamino)benzoic acid,
 3-((6-bromopyridin-3-yl)methyleneamino)benzoic acid,
 2-(4-((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 4-((2-bromopyridin-3-yl)methyleneamino)benzoic acid,
 10 3-((2-bromopyridin-3-yl)methyleneamino)benzoic acid, and
 2-(2-((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid.

The methylamino- and methyleneamino-heteroaryl compounds in the chemical compound library may be prepared using known methods. Suitably, a combinational synthetic
 15 approach based on solventless and/or microwave reaction conditions may be used. These conditions resulted in improved yields and purity of the synthetic products.

In another aspect of the invention there is provided a compound having the formula (III)



(III)

20

wherein

A is a divalent linker selected from the group -(CH₂)₁₋₁₀-, -aryl-, -(CH₂)₁₋₅-aryl-, -(CH₂)₁₋₅-aryl-(CH₂)₁₋₅-, -aryl-(CH₂)₁₋₅-, -aryl-NH-(CH₂)₁₋₅-, -aryl-NH-aryl-, -aryl-CONH-(CH₂)₁₋₅-,
 -aryl-CONH-aryl-, -aryl-CO-(CH₂)₁₋₅-, and -aryl-CO-aryl-, where each aryl is optionally
 25 substituted;

R₁₂ is selected from -CO₂H, -NH₂ and OH;

- 15 -

R₁₃ and R₁₄ are independently selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl) and -NH(C₁₋₆alkyl)₂; or R₁₃ and R₁₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R₁₅; and

- 5 R₁₅ is selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl) and -NH(C₁₋₆alkyl)₂;

with the proviso that the following compounds are excluded:

- 2-(pyridin-2-ylmethyleamino)benzoic acid,
4-(pyridin-3-ylmethyleamino)benzoic acid,
10 4-(pyridin-4-ylmethyleamino)benzoic acid,
4-(pyridin-2-ylmethyleamino)benzoic acid,
3-(pyridin-2-ylmethyleamino)benzoic acid,
3-(pyridin-4-ylmethyleamino)benzoic acid,
2-(pyridin-4-ylmethyleamino)benzoic acid,
15 2-(4-(pyridin-4-ylmethyleamino)phenyl)acetic acid,
3-(pyridin-3-ylmethyleamino)benzoic acid, and
2-(4-(quinolin-2-ylmethyleamino)phenyl)acetic acid.

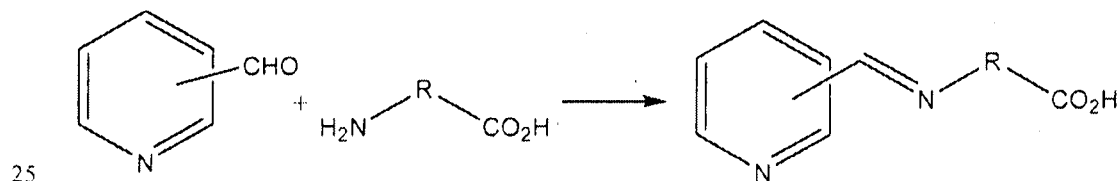
In particular embodiments, the compounds are selected from:

- 20 2-(4-(pyridin-2-ylmethyleamino)phenyl)acetic acid,
4-((pyridin-2-ylmethyleamino)methyl)benzoic acid,
2-(4-chloro-2-(pyridin-4-ylmethyleamino)phenylamino)benzoic acid,
2-(4-chloro-2-(pyridin-2-ylmethyleamino)phenylamino)benzoic acid,
2-(4-(pyridin-3-ylmethyleamino)phenyl)acetic acid,
25 2-(4-(pyridin-4-ylmethyleamino)benzamido)acetic acid,
2-(4-chloro-2-(pyridin-3-ylmethyleamino)phenylamino)benzoic acid,
4-((pyridin-4-ylmethyleamino)methyl)benzoic acid,
6-(pyridin-2-ylmethyleamino)hexanoic acid,
6-(pyridin-3-ylmethyleamino)hexanoic acid,
30 4-(pyridin-3-ylmethyleamino)butanoic acid,
2-(4-(pyridin-3-ylmethyleamino)benzamido)acetic acid,

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- 2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylamino)benzoic acid,
 3-(quinolin-2-ylmethyleneamino)benzoic acid,
 2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylamino)benzoic acid,
 2-(2-((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
 5 2-(4-((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid,
 3-((6-bromopyridin-2-yl)methyleneamino)benzoic acid,
 2-(4-((6-chloropyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(4-((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(2-((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
 10 2-(4-chloro-2-((6-chloropyridin-3-yl)methyleneamino)phenylamino)benzoic acid,
 3-((6-chloropyridin-3-yl)methyleneamino)benzoic acid,
 3-((5-bromopyridin-3-yl)methyleneamino)benzoic acid,
 3-((6-bromopyridin-3-yl)methyleneamino)benzoic acid,
 2-(4-((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 15 4-((2-bromopyridin-3-yl)methyleneamino)benzoic acid,
 3-((2-bromopyridin-3-yl)methyleneamino)benzoic acid, and
 2-(2-((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid.

The compounds where the functionalised group suitable for attachment to a solid support is
 20 a carboxylic acid may be prepared by a general reaction where a nitrogen containing
 heteroaryl carboxylic aldehyde is allowed to react with an amino functionalised aryl- or
 alkyl-carboxylic acid, to generate the corresponding methylene-amino product as shown in
 Scheme 1:



where R is -alkyl- or -aryl-,

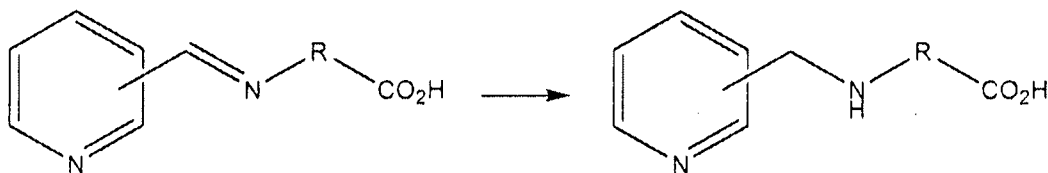
Scheme 1

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For example, suitably substituted 2-, 3- or 4-nitrogen containing heteroaryl carboxylic aldehydes may be mixed with a stoichiometrically equivalent amounts of a suitably substituted 2-, 3- or 4-aminoaryl or aminoalkyl carboxylic acid in the presence of a catalytic amount of acid and the reaction allowed to proceed as a solventless reaction or alternatively as a solution in methanol at room temperature or under microwave/reflux conditions in suitable solvents such as methanol and ethanol. Suitable acids include strong organic acids that are not oxidising and are soluble in a broad range of solvents. Examples include p-toluenesulfonic acid (TsOH), methanesulfonic acid and camphor sulfonic acid, especially TsOH.

10

The imine product, if required, may then be reduced under mild conditions to generate the corresponding methylamino product as shown in Scheme 2:



where R is -alkyl- or -aryl-

15 **Scheme 2**

Suitably the imine may be reduced with a stoichiometric equivalent of a mild reducing agent such in the presence of one stoichiometric equivalent of an acid such as TsOH, in a solventless reaction. Suitable mild reducing agents include catalytic hydrogenation and metal hydride reducing agents such as NaBH₄, NaBH₃CN, (n-Bu)₄NBH₃CN, NaBH₃CN-ZnCl₂, NaBH₃CN-Ti(OiPr)₄, NaBH₃CN-Mg(ClO₄)₂, NaBH₄(OAc)₃, NaBH₄-NiCl₂, NaBH₄-ZnCl₂ (nickel boride), NaBH₄-ZrCl₄, Ti(OiPr)₄-NaBH₄, NaBH₄-H₂SO₄, NaBH₄-wet clay microwave borohydride exchange resin, ZnBH₄, ZnBH₄-ZnCl₂, ZnBH₄-SiO₄, pyridine-borane, picoline-borane, diborane-methanol, decaborane, Zn-AcOH, polymethylhydrosiloxane (PMHS)-Ti(OiPr)₄, PMHS-ZnCl₂, 1-Et₃SiH-CF₃CO₂H, PhMe₂SiH-(C₆F₆)₃, Cl₃SiH-DMF, PhSiH₃-Bu₂SnCl₂, n-Bu₃SnH-DMF, HMPA, n-Bu₃SnH-SiO₂, nBu₂SnH, nBu₂SnClH and PMHS-BuSn(OCOR*)₃ wherein R* is a group that does not destabilize the reducing reagent or result in complex mixtures of byproducts, such as alkyl, alkenyl, cycloalkyl, aryl, heterocyclyl or heteroaryl. In preferred embodiments, the

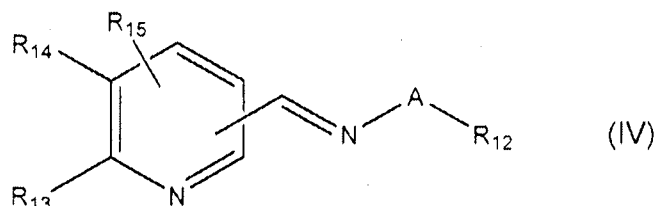
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reducing agent used is NaBH₄. The reducing agent may be selected such that it reduces the C=N bond without reducing other functional groups that may be present in the compound.

The reactions depicted in Scheme 1 and Scheme 2 are also suitable for preparing similar compounds where the functional group suitable for attachment to a solid support is other than a carboxylic acid, for example, a carboxylic anhydride, an acid chloride, an amino group or a hydroxy group.

Thus in another aspect of the invention, there is provided a method of preparing a compound of formula (IV)



wherein

A is a divalent linker selected from the group $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted;

R₁₂ is selected from $-CO_2H$, $-NH_2$ and OH;

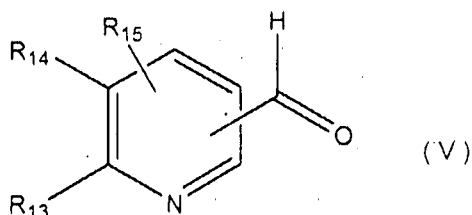
R₁₃ and R₁₄ are independently selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$; or R₁₃ and R₁₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R₁₅; and

R₁₅ is selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$,

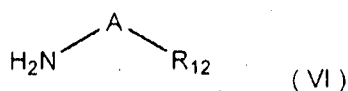
said method comprising

i) grinding a carboxaldehyde of formula (V)

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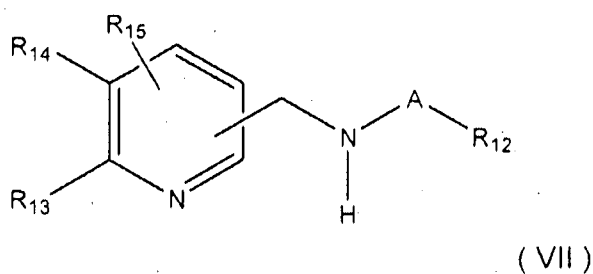
wherein R_{13} to R_{15} are as defined in formula (IV)
together with an amine compound of formula (VI)



wherein A and R_{12} are as defined in formula (IV)
together with a catalytic amount of an organic acid,

- ii) adding solvent in an amount sufficient to form a paste,
- iii) grinding the paste for a time sufficient for reaction to occur, and
- iv) removing the solvent to provide a compound of formula (IV).

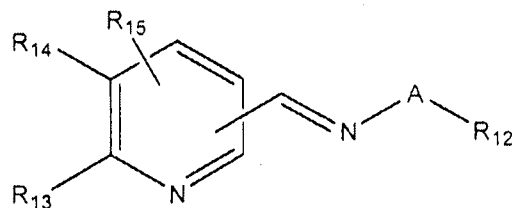
A reduction step to provide a compound of formula (VII):



wherein A, R_{12} , R_{13} , R_{14} and R_{15} are as defined for formula (IV) may also be prepared by similar solventless reduction reaction.

In a further aspect of the invention, there is provided a method of preparing a compound of formula (IV)

- 20 -



(IV)

wherein

A is a divalent linker selected from the group $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$,
 5 $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted;

R_{12} is selected from $-CO_2H$, $-NH_2$ and OH ;

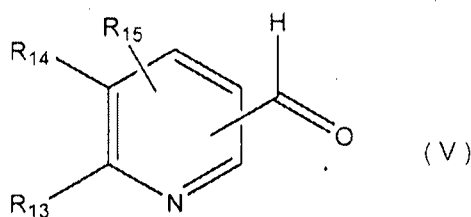
R_{13} and R_{14} are independently selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$; or

10 R_{13} and R_{14} taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R_{15} ; and

R_{15} is selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$

said method comprising:

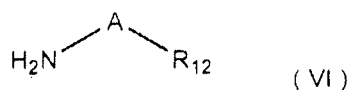
15 i) heating a solution of a carboxaldehyde of formula (V)



(V)

wherein R_{13} to R_{15} are as defined in formula (IV),

20 together with an amine compound of formula (VI)



(VI)

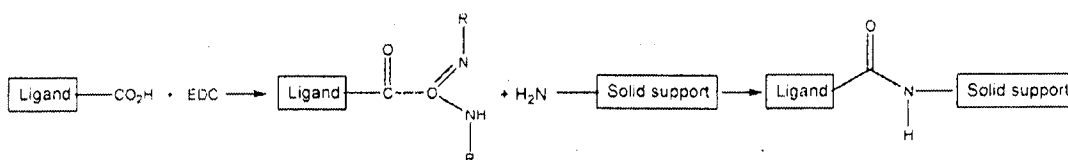
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wherein A and R₁₂ are as defined in formula (IV), together with a catalytic amount of an organic acid, under microwave conditions.

The compounds in the library may be attached to a solid support that has a functional group capable of reacting with the functional group on the compound in the chemical library to form a covalent bond. Suitably, the solid support is functionalised with an amino group, a hydroxy group, a carboxylic acid group, a carboxylic anhydride or an acid chloride. For example, when the compounds in the library are carboxylic acids, the functional group on the solid support is an amino group or a hydroxy group, or when the compound is an amine, the functional group on the solid support is a carboxylic acid, a carboxylic anhydride or an acid chloride, or when the compound is an alcohol, the functional group on the solid support is a carboxylic acid, a carboxylic anhydride or an acid chloride.

The compounds of the chemical library may be immobilised onto the solid support by known reactions for forming amides and esters.

For example, amide formation using activation of the carboxylic acid with a reagent to form an acid chloride or with a suitable carbodiimide reagent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dicyclohexylcarbodiimide (DCC) to form a reactive O-acyl isourea ester. Other suitable coupling agents are also known, for example N,N'-carbonyldiimidazole (CDI). These reactive or activated intermediates readily react with an amino group to form an amide bond as shown in Scheme 3:



Scheme 3

Similar reaction where the activated carboxy group is reacted with a hydroxy functionalised solid support to provide an ester linkage may be effected with the same

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reagents. Alternatively, the carboxyl group may be activated for reaction with an amino group or hydroxy group by reaction with a triazine-based coupling reagent such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium (DMTMM) tetrafluoroborate.

- 5 Similar activation of a carboxylic acid as described above, wherein the carboxylic acid is present on a carboxy-terminated solid support, is suitable for reaction with a ligand that includes an amino or hydroxy functional group for attachment to the solid support.

The coupling conditions may be optimised using model systems such as a carboxy-terminated ligand and benzylamine or benzylalcohol.

The chemical compound library of the invention is immobilised on a solid support to form microarray or nanoarray. The compounds may be immobilised on the solid support in any manner that is robust enough to withstand the washing and handling conditions required for the use of the chemical library. For example, the compound may be immobilised on the solid support by adsorption, such as by ionic interactions, electrostatic interactions and hydrogen bonding. In other embodiments the compounds are immobilised on the solid support by covalent bonding. In some embodiments, the compounds in the chemical array are immobilised on the solid support by the same means, such as covalent bonding. In other embodiments, at least some of the compounds in the chemical array are immobilised by varying means, such as some compounds may be immobilised by hydrogen bonding and some compounds may be immobilised by covalent bonding. In particular embodiments, the compounds are immobilised on the solid support by covalent bonding.

- 25 Suitably functionalised solid supports are known in the art. Any solid support that provides the required functionalised surface on at least part of the support is suitable for use in the invention. For example, the solid support may be glass which has been coated with a layer or layers of functionalised coating. The solid support may be a plastic material that includes surface functional groups or may be reacted with molecules containing the required functional groups to provide a functionalised surface. Suitable polymers include polyacrylates, polycarbonates, polystyrenes, fluorine containing

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polymers, polyethylenes and their derivatives. Examples of suitable polymers include polymethylmethacrylate (PMMA), polyacrylic acid, polyacrylonitrile, polymethacrylate, styrene-acrylonitrile copolymers, butadiene-styrene copolymers and polyalkylstyrenes. The solid support may also be a suitably functionalised silicon chip.

5

The functionalised solid support may have functional groups directly bound to its surface or the functional groups may be attached to the solid support via a linker. For example, the amino groups of amino-functionalised solid support may be directly attached to the solid support surface or attached to the solid support through a linker.

10

In one embodiment, the solid support is in the form of a bead or particle, especially a colloidal bead or particle. Suitably functionalised colloidal beads/particles are commercially available. The beads/particles can be nanoparticles or microparticles selected from a polymer material, an inorganic material, a silicon material, a titania, zirconia or ceramic material, a quartz material, a glass material, a magnetic material, and combinations thereof. For example amino-terminated or carboxy-terminated melamine microspheres are available from Corpuscular, Inc. (Cold Spring, NY, USA) as a 5% by weight aqueous colloidal solution. Other commercially available functionalised beads/particles are available from Estapor-Merck Chimie s.a.s (F-94126 Fontenay Sous Bois Cedex France) and Sigma-Aldrich (St. Louis, MO, USA). Zirconia, titania and composite ceramic-based unmodified or functionalised materials may also be used as solid supports. Other hydroxy, amino and carboxy functionalised beads are also commercially available. A person skilled in the art would be able to identify other commercially available functionalised solid supports or solid supports that may be functionalised so that they are suitable for immobilising the chemical compounds libraries of the invention.

In some embodiments of the invention, at least one compound from the chemical compound library is immobilised on a solid support having a profiled surface. For example, the solid support may have discrete zones of functionalisation suitable for binding to the compounds of the chemical library. These discrete zones of functionalisation may occur on a flat surface, prepared for instance, by known lithographic

30

- 24 -

and masking techniques. The discrete zones of functionalisation may also occur in wells or channels or on raised surfaces, prepared for example by photolithography or ablation.

In one embodiment functionalised beads or particles upon which compounds of the chemical compound library have been immobilised may be trapped in the profiled surface of an array structure preferably having a grid pattern.

A suitable array is described in WO 2008/000031, the contents of which are incorporated herein by reference. In particular, the beads upon which at least one compound of the chemical compound library is immobilised may be trapped in the surface indentations of a mould. Once trapped, a solidifiable material is poured onto the mould to cast an array. Once removed from the mould, the array provides the at least one chemical compound immobilised on a bead positioned at the top of a raised profiled feature such as a frustopyramidal or frustoconical shaped post, the truncated apex of which has the bead embedded within it.

The array may be a nanoarray or a microarray. The raised profiled features may be nanostructures or microstructures. The beads carrying the immobilised chemical library may be applied to the mould and randomly trapped in the mould indentations. The random trapping can include spin-coating-directed deposition of a colloidal solution of the beads or particles. The random trapping can be at least partially influenced by selectively varying at least one of the spin speeds of the spin-coating-directed deposition, the concentration of the colloidal solution, the shape and size of the particles, and the shape, width, depth and mutual spacing of the surface indentations. Alternatively, a bead or beads may be placed in a specific indentation or beads may be placed in a series of indentations. In some embodiments beads, each having a different chemical compound from the chemical compound library immobilised thereon, may be placed or trapped in different indentations or beads containing a different chemical compound from the chemical library immobilised thereon, may be trapped or placed in the same indentation.

- 25 -

Whether randomly trapped or specifically placed, the spatial positions of the beads carrying the immobilised chemical compounds may represent a unique code which may be used to encode information about the array. For example, different special arrangement in beads in the array may encode information relating to the sample being tested, such as
5 concentration or dilution, the date upon which tests were carried out, the patient or animal from which the sample is derived, or the bacteria that may be identified by the array. The term "informationally-addressable", as used herein, refers to the ability of the profiled features to encode information about an array or an assay.

10 In the case of the random trapping of beads carrying the immobilised chemical compounds, the spatially or informationally-addressable pattern of beads may be detected using label-based detection such as biotin/streptavidin or detection using a luminescent, phosphorescent, fluorescent or radioactive label or dye. A suitable dye includes amino reactive Alexa Fluor® 546. Such a dye reacts with free amino groups present on the bead
15 or solid support and can indicate the presence of beads that do not have library compound immobilised thereon or the presence of beads having an immobilised compound with a free amino group. Alternatively, the spatially or informationally-addressable pattern may be detected using non-label based detection of the immobilised particles such as scanning electron microscopy (SEM), atomic force microscopy (AFM) or confocal (fluorescence)
20 microscopy.

In the case of placement of beads to provide a known pattern, the beads may be placed individually in a desired pattern in the mould indentations. This may be achieved by masking specific indentations to prevent a bead entering that indentation when using spin-
25 coating-directed deposition, or by specific placement of a bead in an indentation. Placement of beads in specific locations may be achieved by atomic force microscopy (AFM) deposition methods and using various types of inverse lithography.

The mould can be made from a rigid substrate material selected from a polymer material,
30 an inorganic material, a silicon material, a quartz material, a glass material, and combinations thereof. For example, the mould can be a silicon substrate. The surface

- 26 -

indentations in the mould may be made by any suitable method, for example, lithography or etching. Furthermore the size of the indentations may vary depending on the detection method used in the assay for which the array is to be used. Labelling detection may allow small indentations to be used as small as 2-10 nm, whereas mass spectrometry may require
5 a cluster of beads at each detection site, typically a few mm such as 1-2 mm in size, to ensure the laser is able to focus on the sample.

The solidifiable material can be selected from a polymeric material, a polymerisation initiator, a polymerisation catalyst, an inorganic precursor, a metal precursor, and
10 combinations thereof. For example, the solidifiable material can be a polymeric material such as polydimethylsiloxane (PDMS).

Once the solidifiable material is set, the array bearing the beads at the top of the raised posts can be removed from the mould and is ready for use in an assay.

15

In another aspect of the present invention there is provided an assay for detecting the presence of one or more bacteria in a test sample comprising:

i) contacting a chemical compound array comprising at least one compound of formula (IA) as defined above, with a test sample that is suspected of containing a bacterial
20 peptide or protein that interacts with at least one chemical compound in the chemical compound array; and

(ii) detecting the interaction between the bacterial peptide or protein and the at least one chemical compound in the array.

25 In preferred embodiments, the chemical compound array comprises at least one compound of formula (IIA) as defined above.

The bacterium or bacteria to be detected may be any bacterium or bacteria and will depend on the object of the assay. The bacteria may be pathogenic or beneficial and may also be a
30 cultivar or strain of bacteria. Examples of bacteria include Gram positive or Gram negative bacteria, especially Gram positive bacteria including bacteria of the Genus

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Bacillus (e.g. *B. subtilis*, *B. anthracis*, *B. cereus*, *B. firmis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. coagulans*, *B. pantothenicus*, *B. alvei*, *B. brevis*, *B. circulans*, *B. laterosporus*, *B. macerans*, *B. polymyxa*, *stearothermophilus*, *B. thuringiensis*, *sphaericus*), *Staphylococcus* (e.g. *S. aureus*, *S. epidermidis*, *S. haemolyticus*,
 5 *S. saprophyticus*), *Streptococcus* (e.g. *S. pyogenes*, *S. pneumoniae*, *S. agalactiae*,
S. pyogenes, *S. agalactiae*, *S. dysgalactiae*, *S. equisimilis*, *S. equi*, *S. zooepidemicus*, *S.*
anginosus, *S. salivarius*, *S. milleri*, *S. sanguis*, *S. mitior*, *S. mutans*, *S. faecalis*, *S. faecium*,
S. bovis, *S. equinus*, *S. uberis*, *S. avium*), *Aerococcus*, *Gemella*, *Corynebacterium*, *Listeria*,
Kurthia, *Lactobacillus*, *Erysipelothrix*, *Arachnia*, *Actinomyces*, *Propionibacterium*, *Rothia*,
 10 *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Nocardia* and *Mycobacterium*.

A particular bacterium or strain of a bacterium may be identified with a specific disease or infection to be detected. For example, if the assay was to detect bovine mastitis, the bacteria to be detected may include *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S.*
 15 *uberis* which have all been implicated in bovine mastitis in dairy cows.

In this case, not only is the assay able to provide confirmation that a bacterial infection is present but may also provide information relating to the specific bacterium or bacteria or strains of a bacterium that is causing the infection. This may lead to better identification of
 20 a treatment, for example, the most suitable antibiotic to treat the infection.

Each bacterial species includes proteins, polypeptides and peptides which are characteristic of that species, or even a strain of the species. The following criteria may be employed to identify a marker protein, polypeptide or peptide characteristic of a particular bacterium:

- 25 a) the target protein(s) is(are) relatively abundant in bacteria cells;
- b) the genome sequence and/or proteome of the bacterium was known;
- c) the expression of the target protein(s) must be essential to cell growth, survival or reproduction, possibly belonging to particular functional group such as cell wall biosynthesis, protein biosynthesis (including the entire tRNA synthetase
 30 complex), fatty acid biosynthesis, DNA replication or RNA transcription;

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- d) the target protein(s) must have homologues in other target bacteria, but nevertheless is structurally unique;
- e) the target protein(s) must have sites within its amino acid sequence that are proteolytic accessible;
- 5 f) the target proteins and some proteolytically-derived fragment(s) thereof must be unique for a particular bacterium;
- g) the target proteins and some proteolytically-derived fragment(s) thereof must have specific binding behaviour for individual ligands, which are immobilised onto nanosized beads, which can be supported in the array format as described in this application; and
- 10 h) the target proteins or the proteolytically-derived fragment(s) may be detectable in MALDI ToF mass spectrometry.

To identify specific markers, biological samples containing bacterial cells are lysed and the cell lysate recovered. The protein concentration of the lysates is then determined by
15 known methods such as BCA protein assay. The lysate protein is then precipitated and digested to provide polypeptides and peptides. Digestion may be carried out with any suitable proteolytic enzyme or enzymes, for example, trypsin and chymotrypsin or mixtures thereof. Commercially available RapiGest™ SF may be used. Moreover,
20 digestion can be achieved using one of a number of established methods of chemical cleavage with suitable chemical reagents such as cyanogen bromide, formic acid, trifluoroacetic acid, etc.

The characteristic peptides of each bacterium may be identified by mass spectrometry and
25 comparison with databases of bacterial proteins will allow identification of parent proteins and the bacterium or strain of bacterium of which they are characteristic.

Each test sample is also subject to lysis of bacterial cells and digestion of peptides so that characteristic peptides are produced.

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In the assay of the invention, the chemical compound array is contacted with a test sample suspected of containing a protein, polypeptide or peptide of bacterium or bacteria to be detected. Alternatively, the test sample may be a control sample that contains a known amount of the bacterial protein, polypeptide or peptide.

5

The contact may be achieved by subjecting an array or a specific bead or zone in the array to an aliquot of test sample. Alternatively, an entire array may be soaked or submerged in a solution of test sample. Contact may also be achieved by spraying technologies such as ink-jet spraying technologies.

10

Several dilutions of test sample may be used.

The arrays may then be washed at least once to remove excess and/or unreacted test sample. The array may then be subject to detection for peptides that have reacted with a chemical compound in the immobilised chemical library (positive mode). The washings
15 may also be retained and analysed to detect depletion of particular peptide (negative mode) compared to a control sample, either not exposed to beads at all or exposed to beads that do not have immobilised chemical compounds from the chemical compound library.

20 Detection is suitably achieved by label detection such as biotin/streptavidin, radiolabeling, or a fluorescent, phosphorescent, or luminescent label or dye that reacts with the peptide or protein bound to the assay array. Such detection provides information relating to whether there is a peptide bound or not but does not provide structural information to enable identification of the peptide. In a preferred embodiment, detection is achieved by mass
25 spectrometry, for example Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry. (MALDI TOF MS). MALDI TOF MS may not only provide information relating to the presence of bound bacterial peptide, but also structural information relating to the bound peptide.

30 For example, in the positive mode of the assay, the beads with immobilised compounds from the chemical library and potentially peptides from the test sample are subject to mass

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spectrometric detection or detection by use of a label based detection system such as biotin/streptavidin, or a fluorescent, luminescent or phosphorescent label or a radio label. In this mode, peptides that are bound to the compounds of the chemical library are identified, either by their presence on the array or by molecular mass when mass spectrometric detection is used.

If different compounds in the chemical compound library are immobilised on the solid support and are arranged in a known spatially and informationally-addressable pattern, and the binding affinity of the bacterial protein, polypeptide or peptide for a particular compound or group of compounds in the library and which are part of the spatially- and informationally-addressable array, the presence of a particular protein, polypeptide or peptide may be identified by its binding pattern on the array. The bacteria from which the peptide is known to be derived may therefore be identified.

In the negative mode, the washings may be subject to mass spectrometric analysis or by use of antibodies, particularly monoclonal antibodies that have a specificity for the marker peptides linked to a suitable amplification enzyme. The absence of peptides characteristic of a particular bacterium or strain of bacterium may be identified. This mode may be used alone, or may be used to confirm the results of positive mode testing.

Control samples may include test sample exposed to beads which do not have compounds from the chemical library immobilised thereon. This control may be used to identify any non specific binding that may occur on the beads. This control sample may also be analysed in both positive and negative form.

Another control sample that may be used is a test sample not exposed to any array or library compounds. This control sample should provide a full complement of peptides in the test sample.

When the arrays are intended to be used in conjunction with matrix assisted laser desorption ionisation time-of-flight mass spectrometry instrumentation, the functionalised

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array must have spots or areas of functionalisation of at least 0.5 mm to enable adequate focussing of the laser. Functionalised beads containing immobilised compounds or lacking immobilised compounds may be deposited on surfaces or at the bottom of a mould surface indentation of suitable size as a bead cluster, or as multiple bead clusters.

5

In one embodiment, the assay is for detection of *E. coli*. In this embodiment, the bacterial peptides that characterise the *E. coli* and bind to compounds in the chemical compound library include:

LEVVVNER (m/z ion 957.1385) [SEQ ID NO: 1]

10 YSYVDENGETK (m/z ion 1304.564) [SEQ ID NO: 2]

REEESAAAAEVEER (m/z ion 1575.725) [SEQ ID NO: 3] and

EMLIADGIDDNELLNSLAAVK (m/z ion 2226.169) [SEQ ID NO: 4].

15 In another embodiment of the invention, there is provided a use of a bacterial peptide selected from:

LEVVVNER (m/z ion 957.1385) [SEQ ID NO: 1]

YSYVDENGETK (m/z ion 1304.564) [SEQ ID NO: 2]

REEESAAAAEVEER (m/z ion 1575.725) [SEQ ID NO: 3] and

EMLIADGIDDNELLNSLAAVK (m/z ion 2226.169) [SEQ ID NO: 4]

20 as a bacterial marker that characterises *E. coli* or a strain of *E. coli*.

In some embodiments, the bacterial infection being detected with the assay is bovine mastitis. The sample may be obtained by any suitable means, for example, from a sample of milk or by taking swab from the infected area.

25

In another aspect of the invention there is provided a method of detecting bacteria in a test sample comprising:

- i) providing a chemical compound array defined above;
- ii) contacting the array with a biological test sample of interest,
- 30 iii) determining any interaction of a bacterial peptide marker in the test sample with a chemical compound in the chemical compound array, and

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iv) identifying the bacterial peptide marker and the bacterium from which the bacterial marker is derived.

In yet another aspect of the invention there is provided a method of detecting a bacterium
5 responsible for bovine mastitis comprising:

- i) providing a chemical compound array comprising a compound of formula (IIA);
- ii) contacting the array with a bovine test sample of interest,
- iv) determining any interaction of a bacterial peptide marker in the test sample
10 with a chemical compound in the chemical compound array, and
- v) identifying the bacterial peptide marker and the bacterium from which it is derived.

In order that the nature of the present invention may be more clearly understood and put
15 into practical effect, particular preferred embodiments thereof will now be described with reference to the following non-limited examples and Figures.

Description of the Figures

Figure 1 is a schematic diagram for the fabrication of the PDMS derived beads-on-posts
20 microarray.

Figure 2 is a close-up microscopic image of a bead trapped in a pyramidal well (a) and at the apex of a PDMS pyramid (b). Note the clear boundary between the PDMS polymeric material and the bead. The bars represent 2.5µm.

Figure 3 is an image of an example of a fabricated microarray with (a) the beads randomly
25 deposited in the wells on the silicon wafer, and (b) the beads positioned at the apex of the complementary PDMS pyramids/posts of the microarray. The bar represents 5 µm.

Figure 4 is a general schematic for the fabrication of microarray with functionalized bead clusters deposited in a 5 x 5 mm grid arrangement.

Figure 5 provides microscopic images of immobilised bead clusters of *ca.* 1 mm in
30 diameter. Images were collected using DIC optics and at (A) 20 x (top left) or (B) 60 x (top

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right) magnification. Also shown are the microscopic images (DIC optics, 60 x magnification) of the immobilised bead clusters (C, bottom left) before and (D, bottom, right) after sonication. Individual beads are approximately 2 μm in diameter.

Figure 6 demonstrates alternative geometric codes (a, b and c) obtained through the random deposition of beads in wells. The bar represents 10 μm .

Figure 7. Fluorescent images of the bead microarray before (a) and after (b) the reaction of the amino-bead-PDMS microarray with a solution of the amino-reactive dye Alexa Fluor® 546. The bar represents 10 μm .

Figure 8 is a schematic diagram of the general format that can be used to interrogate the various bead systems bearing different immobilised chemical compounds or bead systems that have not been chemically functionalised. In the shown format, beads having different chemical compounds from the library immobilised on them are arrayed in the X-direction as Bead 1, 2, 3,, N-1, N, whilst samples of different dilution/concentrations are arrayed in the X-direction, as Sample 1, 2, 3,, M. The location of the different beads and dilution regimes can be specified via the X-Y codes, such as 11, 12, 13 ...1N or 11, 21, 31.....1M. In the Y-direction manipulations other than dilution can be affected, i.e. different washing or buffer handling steps can be employed. Depending on the specific MALDI-TOF-MS instrumentation employed and the laser focal width, sample plates of 5 x 5 cm dimensions and containing from 4 through 1400 immobilised bead clusters, can be used. A random distribution of beads can also be achieved where a 100% occupancy of the PDMS polymer surface sites is not utilized, permitting the groups of arrayed beads, bearing the same or different chemical compounds, to be read separately from the information carried by each bead. As such, in this configuration, the arrayed beads represent a self-identifying bar code.

Figure 9 provides MALDI-TOF mass spectra in the m/z range of 500 to 2500 from three experiments after incubation of the tryptic peptides from an *E. coli* preparation at 1:10 dilution with (A) beads containing the immobilised compound 4-(pyridin-4-ylmethyleneamino)benzoic acid (7) with the test sample analysed in the adsorption mode, (B) unmodified beads as negative control, and (C) buffer alone as positive control. Comparison of the three spectra revealed significant differences in the signal intensities for particular signals indicating the occurrence of peptide-immobilised ligand binding events.

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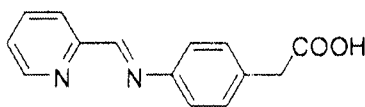
Such differences occur, for example, in the region under the arrow in the m/z range of 800-1100.

Figure 10 is an expanded view of the MALDI TOF mass spectra in the m/z range of 800-1100 from three experiments after incubation of tryptic peptides from an *E. coli* preparation at 1:10 dilution with (A) beads containing the immobilised ligand, 4-(pyridin-4-ylmethyleneamino)-benzoic acid (7) as test sample for the positive adsorption mode, (B) unmodified beads as negative control and (C) buffer as positive control. Comparison of the spectrum (A) with spectra (B) and (C), respectively, reveal significant differences in the signal intensities (the location of the $m/z = 957.1385$ $[M+H]^+$ signal is identified by the arrow).

EXAMPLES

Example 1: Synthesis of 2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid (1)

Pyridine-2-carboxaldehyde (0.45 g, 4.2 mmol) was dissolved in methanol (*ca.* 30 mL) and the mixture stirred at room temperature. To this mixture, 4-amino-phenylacetic acid (0.64 g, 4.2 mmol) and a catalytic amount of p-toluenesulfonic acid (TsOH, 6 mg) were added and the resulting suspension stirred at room temperature with the reactants dissolving to give an orange solution. The reaction mixture was left to stir for 15 hours at room temperature, yielding an orange suspension. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to leave the product as an orange solid. Yield 42%.



TLC: (MeOH:CH₂Cl₂ 10:90) 2 spots R_f 0.50 and 0.46.

MS $[M + H]^+$ m/z 241.2.

¹H NMR (300 MHz, d₆-DMSO) δ 3.65 (2H, s, CH₂), 7.31 (4H, m, Ar-H), 7.53 (1H, t, $J = 4.2$ Hz, Ar-H), 7.95 (1H, t, $J = 7.8$ Hz, Ar-H), 8.53 (1H, d, $J = 7.8$ Hz, Ar-H), 8.60 (1H, s, CH=CN), 8.71 (1H, d, $J = 4.2$ Hz, Ar-H).

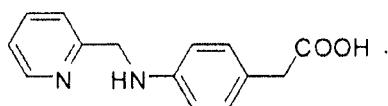
Example 2: Solventless synthesis of 2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid (1)

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Pyridine-2-carboxaldehyde (0.91 g, 8.5 mmol) and 4-aminophenylacetic acid (1.28 g, 8.5 mmol) were placed in a mortar and pestle to which a catalytic amount of TsOH (10 mg) was added. The mixture was ground at room temperature for approximately 10 minutes. Dry methanol (1 mL) was added and the paste was ground for a further 20 minutes. The solid was removed from the mortar, placed into a round bottom flask and dried under high vacuum to give the final product as a yellow coloured solid. Yield 90%. The MS and NMR spectroscopic data for this compound were identical to that obtained in Example 1.

Example 3: Synthesis of 2-(4-(pyridin-2-ylmethylamino)phenyl)acetic acid (2)

A mixture of 4-(pyridin-2-ylmethylamino)benzoic acid (1.20 g, 5 mmol), sodium borohydride (0.189 g, 5 mmol) and TsOH (0.951 g, 5 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon disappearance of the starting material the reaction was stopped. Then, saturated sodium bicarbonate (~ 10 mL) was added the solution was poured into a beaker. The pH of the solution was carefully acidified to pH ~4.5 by treating with 5 M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give the product (2), as a yellow solid, which could be recrystallised from ethanol. Yield 92%.



TLC (MeOH:CH₂Cl₂: 10:90) 1 spot, R_f: 0.25.

MS [M+H]⁺ m/z 243.1.

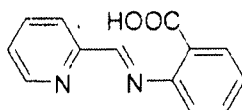
¹H NMR (400 MHz, d₆-DMSO) δ 3.43 (2H, s, CH₂), 4.33 (2H, d, *J* = 5.6 Hz, N-CH₂), 6.22 (1H, s, NH), 6.50 (2H, d, *J* = 8.5 Hz, Ar-H), 6.93 (2H, d, *J* = 8.5 Hz, Ar-H), 7.23 (1H, dt, *J*₁ = 3.2 Hz, *J*₂ = 1.5 Hz, Ar-H), 7.34 (1H, d, *J* = 3.2 Hz, Ar-H), 7.71 (1H, dt, *J*₁ = 4.8 Hz, *J*₂ = 1.5 Hz, Ar-H), 8.51 (1H, d, *J* = 4.8 Hz, Ar-H).

Example 4: Synthesis of 2-(4-(pyridin-2-ylmethyleamino)benzoic acid (3)

Pyridine-2-carboxaldehyde (0.96 g, 8.97 mmol) was dissolved in methanol (ca. 30 mL) and the solution stirred at room temperature. To this solution, anthranilic acid (1.23 g, 8.97 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature with the reactants dissolving to give an orange

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solution. Upon further stirring for 15 hours, no visible change in colour was noted. The solution was concentrated in vacuo and analysed.



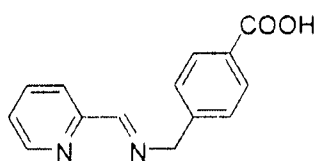
5 TLC (MeOH:CH₂Cl₂ 10:90) 2 spots R_f 0.45 and 0.42.

MS [M+H]⁺ m/z 227.1.

¹H NMR (300 MHz, d₆-DMSO) an 85:15 mixture of product and starting material.

Example 5: Synthesis of 4-((pyridin-2-ylmethyleneamino)methyl)benzoic acid (4)

10 To pyridine-2-carboxaldehyde (1.06 g, 9.9 mmol) and a catalytic amount of TsOH (10 mg) in a mortar, 4-(methylamino)-benzoic acid (1.0 g, 6.6 mmol) was added slowly in portions. After each portion was added the reactants were ground together with the mortar and pestle. Upon addition of the final portion of the acid the mixture was ground to give a pale pink solid, which solidified to yield a pink powder upon standing. This solid was washed
15 with methanol and dried *in vacuo* to yield (4) as an off white solid. Yield 84%.



TLC (MeOH:CH₂Cl₂ 10:90) 3 spots R_f 0.20, 0.40 (major) and 0.50.

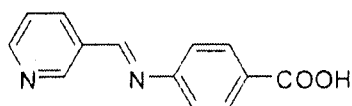
MS [M+H]⁺ m/z 240.9

¹H NMR (400 MHz, d₆-DMSO) δ 4.92 (2H, s, CH₂), 7.47 (3H, m, Ar-H), 7.89 (3H, m, Ar-H), 8.01 (1H, m, Ar-H), 8.53 (1H, s, CH=N), 8.67 (1H, m, Ar-H), 12.70 (1H, br s, COOH).
20 ¹³C NMR (100 MHz, d₆-DMSO) δ 63.08 (CH₂), 120.56 (CH), 125.26 (CH), 127.94 (C), 129.38 (CH), 129.44 (CH), 136.89 (CH), 144.25 (CH), 149.4 (C), 153.97 (C), 163.31 (C=N), 167.13 (COOH).

25 **Example 6:** Synthesis of 4-(pyridin-3-ylmethyleneamino)benzoic acid (5)

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Pyridine-3-carboxaldehyde (1.34 g, 12.52 mmol) was dissolved in methanol (~50 mL) and the solution stirred at room temperature. To this mixture, aminobenzoic acid (1.72 g, 12.52 mmol) and a catalytic amount of TsOH (15 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within 30 minutes of dissolving, a yellow precipitate was formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to leave a yellow solid. Yield 90%.



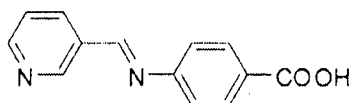
TLC (MeOH:CH₂Cl₂ 10:90) 2 spots R_f 0.45 and 0.42.

10 MS [M+H]⁺ m/z 227.1.

¹H NMR (200 MHz, d₆-DMSO) δ 6.84 (2H, m, Ar-H), 7.26 (1H, m, Ar-H), 7.46 (2H, m, Ar-H), 7.70 (2H, m, Ar-H), 8.58-8.70 (2H, m, Ar-H, CH=N).

Example 7: Synthesis of 4-(pyridin-3-ylmethylamino)benzoic acid (6)

15 A mixture of 4-(pyridin-3-ylmethyleneamino)benzoic acid (1.13 g, 5 mmol), sodium borohydride (0.189 g, 5 mmol) and TsOH (0.951 g, 5 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon disappearance of the starting material the reaction was stopped. Then, saturated sodium bicarbonate (10 mL) was added the solution was poured into a beaker. The pH of the solution was carefully acidified to ~pH 4.5 by treating with 5M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give (6) as a light yellow solid. Yield: 83%.



TLC (MeOH:CH₂Cl₂ 10:90) 1 spot R_f: 0.45

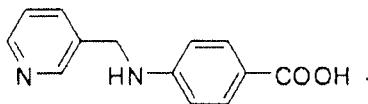
25 MS [M+H]⁺ m/z 229.0.

¹H NMR (400 MHz, d₆-DMSO) δ 4.37 (2H, s, CH₂), 6.61 (2H, d, *J* = 8.4 Hz, Ar-H), 7.03 (1H, s, NH), 7.36 (1H, dt, *J*₁ = 8.4 Hz, *J*₂ = 3.6 Hz, Ar-H), 7.65 (2H, d, *J* = 8.4 Hz, Ar-H), 7.74 (1H, d, *J* = 7.6 Hz, Ar-H), 8.45 (1H, d, *J* = 3.6 Hz, Ar-H), 8.58 (1H, s, Ar-H).

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Example 8: Synthesis of 4-(pyridin-4-ylmethyleneamino)benzoic acid (7)

Pyridine-4-carboxaldehyde (1.18 g, 11.02 mmol) was dissolved in methanol (ca 50 mL) and the mixture stirred at room temperature. To this solution, 4-aminobenzoic acid (1.51 g, 11.02 mmol) and a catalytic amount of TsOH (15 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a couple of minutes of dissolving, a yellow precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product as a yellow solid. Yield >90%.



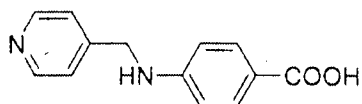
TLC (MeOH:CH₂Cl₂ 10:90) 2 spots, R_f 0.40 and 0.37.

MS [M+H]⁺ m/z 227.1

¹H NMR (200MHz, d₆-DMSO) δ 7.33 (2H, m, Ar-H), 7.58 (1H, m, Ar-H), 7.98 (2H, m, Ar-H), 8.32 (1H, m, Ar-H), 8.72 (2H, m, Ar-H, CH=N), 9.08 (1H, Ar-H).

Example 9: Synthesis of 4-(pyridin-4-ylmethylamino)benzoic acid (8)

In a 2 neck flask 4-(pyridin-4-yl-methyleneamino)benzoic acid (1.47 g, 6.48 mmol) was added to 30 mL of methanol. The solution was warmed to 40 °C with stirring and sodium borohydride (245 mg, 6.48 mmol, 1 eq.) was gradually added over 30 minutes. The mixture was then stirred at reflux for 20 minutes. Subsequently, water (30 mL) was added and the mixture was allowed to cool. Following measurement of the pH of the solution (pH 9.0), the solution was then carefully acidified with 5 M HCl until a pH of pH 4-5 was reached. At this point a precipitate formed, which was filtered through a sinter. The solid was washed with cold methanol and dried *in vacuo* to give the crude product as an orange-yellow solid. Yield: 67 %.

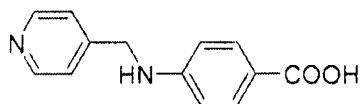


Upon analysis by MS and NMR spectroscopy the crude product was found to be a mixture of 3 different compounds and some starting material. No attempt was made to purify this

mixture further. An alternative methodology to the above solvent-based procedure was developed.

Example 10: Solventless synthesis 4-(pyridin-4-ylmethylamino)benzoic acid (8)

- 5 A mixture of 4-(pyridin-4-ylmethyleneamino)benzoic acid (452 mg, 2 mmol), sodium borohydride (151 mg, 2 mmol) and TsOH (760 mg, 2 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon disappearance of the starting material the reaction was stopped. Then, saturated sodium bicarbonate (10 mL) was added and the solution was poured into a beaker. The pH of the
10 solution was acidified to ~ pH 4.5 by treating with 5 M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give the product (8) as a creamy coloured solid. Yield: 91 %.



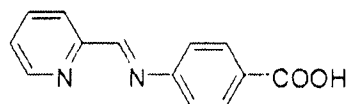
TLC (MeOH:CH₂Cl₂ 10:90) 1 spot, R_f 0.35.

- 15 MS [M+H]⁺ m/z 229.0

¹H NMR (400 MHz, d₆-DMSO) δ 4.41 (2H, s, CH₂), 6.65 (2H, d, *J* = 8.3 Hz, Ar-H), 7.06 (1H, s, NH), 7.40 (1H, d, *J* = 4.4 Hz, Ar-H), 7.73 (3H, m, Ar-H), 8.49 (1H, s, Ar-H), 8.61 (1H, s, Ar-H).

- 20 **Example 11:** Synthesis 4-(pyridin-2-ylmethyleneamino)benzoic acid (9)

Pyridine-2-carboxaldehyde (1.10 g, 10.30 mmol) was dissolved in methanol (*ca.* 50 mL) and the solution stirred at room temperature. To this solution, 4-aminobenzoic acid (1.41 g, 10.30 mmol) and a catalytic amount of TsOH, (14 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a
25 couple of minutes of dissolving, a yellow precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to give the product (9) as a yellow solid. Yield: 88%.



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TLC (MeOH:CH₂Cl₂, 10:90) 3 spots, R_f 0.55, 0.45 and 0.40.

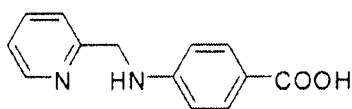
MS [M+H]⁺ m/z 227.1

¹H NMR (200 MHz, d₆-DMSO) δ 7.38 (2H, m, Ar-H), 7.55 (1H, m, Ar-H), 7.99 (3H, m, Ar-H), 8.17 (1H, m, Ar-H), 8.59 (1H, s, CH=N), 8.74 (1H, m, Ar-H), 12.9 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) δ 121.83 (CH), 120.56 (CH), 122.39 (CH), 126.78 (CH), 129.47 (C), 131.43 (CH), 150.61 (CH), 154.48 (C), 155.26 (C), 163.28 (C=N), 167.69 (COOH).

Example 12: Synthesis of 4-(pyridin-2-ylmethylamino)benzoic acid (10)

A mixture of 4-(pyridin-2-ylmethyleneamino)benzoic acid (680 mg, 3 mmol), sodium borohydride (113 mg, 3 mmol) and TsOH (570 mg, 3 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon disappearance of the starting material the reaction was stopped. Then, saturated sodium bicarbonate (10 mL) was added, and the solution was poured into a beaker. The pH of the solution was carefully acidified to ~ pH 4.5 by treating with 5 M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give the product (10) as an orange solid. Yield: 80 %.



TLC (MeOH:CH₂Cl₂ 10:90) 1 spot, R_f 0.30.

MS [M+H]⁺ m/z 229.0

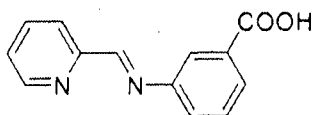
¹H NMR (300 MHz, d₆-DMSO) δ 4.40 (2H, s, CH₂), 6.34 (1H, s, NH), 6.52 (2H, d, *J* = 8.2 Hz, Ar-H), 6.98 (2H, d, *J* = 8.2 Hz, Ar-H), 7.23 (1H, dt, *J*₁ = 3.3 Hz, *J*₂ = 1.5 Hz, Ar-H), 7.29 (1H, d, *J* = 3.3 Hz, Ar-H), 7.68 (1H, dt, *J*₁ = 4.8 Hz, *J*₂ = 1.5 Hz, Ar-H), 8.49 (1H, d, *J* = 4.8 Hz, Ar-H).

Example 13: Synthesis of 3-(pyridin-2-ylmethyleneamino)benzoic acid (11)

Pyridine-2-carboxaldehyde (0.89 g, 8.32 mmol) was dissolved in methanol (*ca.* 40 mL) and the solution stirred at room temperature. To this solution, 3-aminobenzoic acid (1.14 g, 8.32 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting

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suspension stirred at room temperature until all the reactants had dissolved. Within a couple of minutes of dissolving, a yellow/orange precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (11) as a pale yellow solid. Yield: 98%.



TLC (EtOAc:CH₂Cl₂, 1:1) 1 spot, R_f 0.40

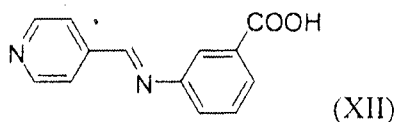
MS [M+H]⁺ m/z 227.1

¹H NMR(400 MHz, d₆-DMSO) δ 7.55 (3H, m, Ar-H), 7.81 (1H, m, Ar-H), 7.86 (1H, m, Ar-H), 7.98 (1H, m, Ar-H), 8.18 (1H, m, Ar-H), 8.63 (1H, s, CH=N), 8.74 (1H, m, Ar-H), 13.05 (1H, br s, COOH).

¹³C NMR (100 MHz, d₆-DMSO) δ 121.99 (CH), 122.38 (CH), 125.76 (CH), 126.37 (CH), 127.87 (CH), 130.20 (CH), 132.57 (C), 137.63 (CH), 150.27 (CH), 151.26 (C), 154.32 (C), 162.47 (C=N), 167.46 (COOH).

Example 14: Synthesis of 3-(pyridin-4-ylmethyleneamino)benzoic acid (12):

Pyridine-4-carboxaldehyde (0.59 g, 5.51 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, 3-aminobenzoic acid (0.76 g, 5.51 mmol) and a catalytic amount of TsOH (8 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a minute of dissolving, a pale yellow precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the recovered solid washed with cold methanol and dried *in vacuo* to yield the product (12) as an off-white solid. Yield: 95 %.



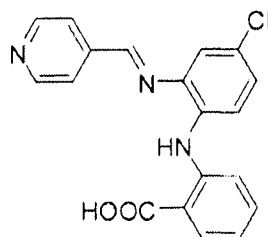
TLC (MeOH:CH₂Cl₂, 10:90) major spot, R_f 0.40, minor spot R_f 0.15.

¹H NMR(400 MHz, d₆-DMSO) δ 7.57 (2H, m, Ar-H), 7.84-7.88 (4H, br m, Ar-H), 8.77 (3H, m, Ar-H, CH=N), 12.10 (1H, br s, COOH).

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Example 15: Synthesis of 2-(4-chloro-2-(pyridin-4-ylmethyleneamino)phenylamino)-benzoic acid (13)

Pyridine-4-carboxaldehyde (0.87 g, 8.12 mmol) was dissolved in methanol (*ca.* 50 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (2.13 g, 8.12 mmol) and a catalytic amount of TsOH (5 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a minute of dissolving, a bright red precipitate formed. The resulting suspension was stirred for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (13) as a red solid. Yield: >85%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.55

MS [M+H]⁺ m/z 352.2

¹H NMR (400 MHz, d₆-DMSO) δ 6.90 (1H, m, Ar-H), 7.30 (1H, m, Ar-H), 7.48-7.59 (4H, br m, Ar-H), 7.94 (3H, m, Ar-H), 8.76 (2H, m, Ar-H), 8.88 (1H, s, CH=N), 10.42 (1H, s, N-H), 13.25 (1H, br s, COOH).

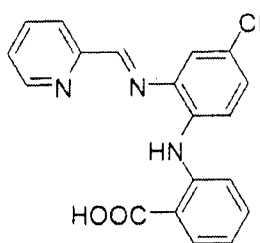
¹³C NMR (100 MHz, d₆-DMSO) δ 115.23 (CH), 115.42 (C), 118.29 (CH), 118.71 (CH), 119.24 (CH), 121.88 (CH), 125.37 (C), 128.12 (CH), 131.67 (CH), 134.55 (CH), 135.97 (C), 139.94 (C), 142.98 (C), 144.91 (C), 150.9 (CH), 160.85 (C=N), 170.21 (COOH).

Example 16: Synthesis of 2-(4-chloro-2-(pyridin-2-ylmethyleneamino)phenylamino)-benzoic acid (14)

Pyridine-2-carboxaldehyde (0.61 g, 5.70 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (1.5 g, 5.70 mmol) and a catalytic amount of TsOH (3 mg)

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were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a minute of dissolving, a bright orange precipitate formed. The suspension was stirred for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (14) as a bright orange solid. Yield: >85%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.65.

MS [M+H]⁺ m/z 352.2

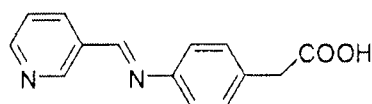
¹H NMR (200 MHz, d₆-DMSO) δ 6.86 (1H, m, Ar-H), 7.25-7.32 (1H, br m, Ar-H), 7.47-7.59 (4H, br m, Ar-H), 7.75 (1H, m, Ar-H), 7.89-7.98 (2H, br m, Ar-H), 8.33 (1H, m, Ar-H), 8.72-8.76 (2H, s, Ar-H, CH=N), 10.40 (1H, s, N-H), 13.01 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) δ 114.28 (CH), 114.41 (C), 117.40 (CH), 117.80 (CH), 118.23 (CH), 121.00 (CH), 124.57 (C), 125.40 (CH), 127.00 (CH), 131.53 (CH), 133.62 (CH), 134.79 (C), 136.56 (CH), 139.61 (C), 144.08 (C), 149.29 (CH), 153.68 (C), 160.15 (C=N), 169.28 (COOH).

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Example 17: Synthesis of 2-(4-(pyridin-3-ylmethyleneamino)phenyl)acetic acid (15)

Pyridine-3-carbox-aldehyde (0.94 g, 8.79 mmol) was dissolved in methanol (*ca.* 40 mL) and the solution stirred at room temperature. To this solution, 4-aminophenylacetic acid (1.33 g, 8.79 mmol) and a catalytic amount of TsOH (10 mg) were added. The suspension
5 was stirred at room temperature. A solution never resulted and the yellow suspension was left to stir for a further 12 hours at room temperature. The reaction was monitored by TLC and was complete within 12 hours. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (15) as a pale yellow solid. Yield: >95%.



10 TLC (MeOH:CH₂Cl₂, 10:90) 3 spots, R_f 0.20, 0.35 and 0.55 (major spot).

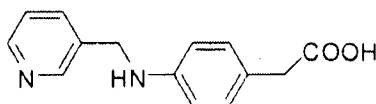
MS [M+H]⁺ m/z 241.1

¹H NMR (200 MHz, d₆-DMSO) δ 3.60 (2H, s, CH₂), 7.23-7.35 (4H, m, Ar-H), 7.54 (1H, m, Ar-H), 8.30 (1H, m, Ar-H), 8.71 (2H, m, Ar-H, CH=N), 9.06 (1H, m, Ar-H), 12.29 (1H, br s, COOH).

15 ¹³C NMR (50 MHz, d₆-DMSO) δ 40.17 (CH₂), 120.96 (CH), 124.02 (CH), 130.23 (CH), 131.56 (C), 133.37 (C), 134.90 (CH), 149.51 (C), 150.37 (CH), 151.87 (CH), 158.15 (C=N), 172.64 (COOH).

Example 18: Synthesis of 2-(4-(pyridin-3-ylmethylamino)phenyl)acetic acid (16)

20 A mixture of 4-(pyridin-3-ylmethyleneamino)benzoic acid (240.0 mg, 1 mmol), sodium borohydride (37.8 mg, 1 mmol) and TsOH (190.2 mg, 1 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon disappearance of the starting material the reaction was stopped. Then, saturated sodium bicarbonate (10 mL) was added, and the solution was poured into a beaker. The pH of the
25 solution was carefully acidified to ~ pH 4.5 with 5 M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give the product (16) as a yellow solid. Yield: 85 %



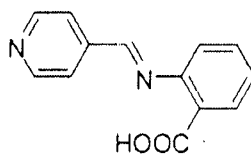
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TLC (MeOH:CH₂Cl₂ 10:90) 1 spot, R_f 0.45.MS [M+H]⁺ m/z 243.1

¹H NMR (200 MHz, d₆-DMSO) δ 3.69 (2H, s, CH₂), 4.35 (2H, d, *J* = 4.4 Hz, CH₂), 6.51 (2H, d, *J* = 8.1 Hz, Ar-H), 6.93 (1H, s, NH), 7.26 (1H, dt, *J*₁ = 8.4 Hz, *J*₂ = 3.5 Hz, Ar-H),
5 7.44 (2H, d, *J* = 8.4 Hz, Ar-H), 7.74 (1H, d, *J* = 7.6 Hz, Ar-H), 8.45 (1H, d, *J* = 3.5 Hz, Ar-H), 8.51 (1H, s, Ar-H).

Example 19: Synthesis of 2-(pyridin-4-ylmethyleamino)benzoic acid (17)

Pyridine-4-carboxaldehyde (0.51 g, 4.77mmol) was dissolved in methanol (*ca.* 30 mL) and
10 the solution stirred at room temperature. To this solution, anthranilic acid (0.65 g, 4.77 mmol) and a catalytic amount of TsOH (6 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within 30 minutes, a pink/brown precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in*
15 *vacuo* to yield the product (17) as an off white solid. Yield: >85%.

TLC (MeOH:CH₂Cl₂, 10:90) R_f 0.40 and 0.50 (major spot).MS [M+H]⁺ m/z 227.1

¹H NMR (200 MHz, d₆-DMSO) δ 6.94 (2H, s, Ar-H), 7.48 (1H, m, Ar-H), 7.60 (2H, m, Ar-H), 7.74 (1H, m, Ar-H), 7.94 (1H, m, Ar-H), 8.71 (2H, m, Ar-H, CH=N), 12.45 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 122.20 (CH), 124.26 (CH), 126.84 (C), 127.88 (CH), 129.14 (CH), 134.89 (CH), 143.28 (CH), 149.60 (C), 153.657 (C), 164.06 (C=N), 167.87 (COOH).

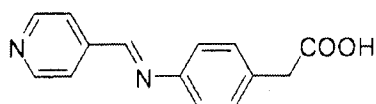
25

Example 20: Synthesis of 2-(4-(pyridin-4-ylmethyleamino)phenyl)acetic acid (18)

Pyridine-4-carbox-aldehyde (0.89 g, 8.32 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this mixture, 4-amino-phenylacetic acid (1.26 g, 8.32 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting

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suspension stirred. Whilst stirring, the pale yellow suspension changed colour to a bright yellow over 12 hours at room temperature. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (18) as a bright yellow solid. Yield: >85%.



5

TLC (MeOH:CH₂Cl₂, 10:90) R_f 0.15, 0.20 and 0.25 (major spot).

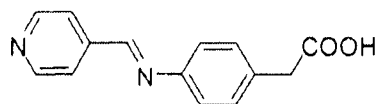
MS [M+H]⁺ m/z 241.1

¹H NMR (200 MHz, d₆-DMSO) δ 3.61 (2H, s, CH₂), 7.30 (4H, m, Ar-H), 7.84 (2H, m, Ar-H), 8.73-8.87 (3H, m, CH=N, Ar-H), 12.62 (1H, br s, COOH).

10 ¹³C NMR (50 MHz, d₆-DMSO) 40.14 (CH₂), 121.12 (CH), 122.14 (CH), 130.29 (CH), 133.91 (C), 142.51 (C), 148.99 (C), 150.44 (CH), 158.85 (C=N), 172.58 (COOH).

Example 21: Solventless synthesis of 2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid (18)

15 Pyridine-4-carboxaldehyde (0.91 g, 8.5 mmol) and 4-amino benzoic acid (1.28 g, 8.5 mmol) were placed in a mortar and pestle to which a catalytic amount of TsOH (10 mg) was added. The mixture was ground at room temperature for approximately 10 minutes. Then, dry methanol (1 mL) was added and the paste was ground for a further 20 minutes. The solid was transferred from the mortar into a round bottom flask and dried under high
20 vacuum to give the final product (18) as a light yellow solid. Yield: 92 %.



TLC (MeOH:CH₂Cl₂ 10:90) 1 spot, R_f 0.10.

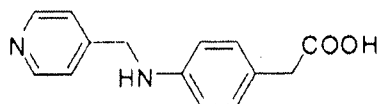
MS [M+H]⁺ m/z 241.0

25 ¹H NMR (300 MHz, d₆-DMSO) δ 3.64 (2H, s, CH₂), 7.31 (4H, m, Ar-H), 7.83 (2H, d, J = 6.0 Hz, Ar-H), 8.70 (1H, s,), 8.53 (1H, d, J = 7.8 Hz, Ar-H), 8.70 (1H, s, CH=N), 8.74 (2H, d, J = 6.0 Hz, Ar-H).

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Example 22: Synthesis of 2-(4-(pyridin-4-ylmethylamino)phenyl)acetic acid (19)

A mixture of 2-(pyridin-4-ylmethyleneamino)benzoic acid (1.20 g, 5 mmol), sodium borohydride (0.189 g, 5 mmol) and TsOH (0.951 g, 5 mmol) was ground in a mortar and pestle at room temperature for 15 minutes. The reaction was monitored by TLC and upon
5 disappearance of the starting material the reaction was stopped. Saturated sodium bicarbonate (~ 10 mL) was added, and the solution poured into a beaker. The pH of the solution was carefully acidified to ~ pH 4.5 by treating with 5 M HCl and a precipitate formed. The precipitate was filtered off and dried *in vacuo* to give the product (19) as a creamy coloured solid. Yield: 91 %.



10

MS [M+H]⁺ m/z 243.1

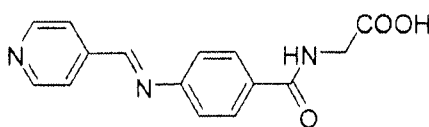
¹H NMR (200 MHz, d₆-DMSO) δ 3.33 (2H, s, CH₂), 4.29 (2H, d, *J* = 5.0 Hz, N-CH₂), 6.32 (1H, s, NH), 6.47 (2H, d, *J* = 8.2 Hz, Ar-H), 6.92 (2H, d, *J* = 8.2 Hz, Ar-H), 7.33 (2H, d, *J* = 4.6 Hz, Ar-H), 8.47 (2H, d, *J* = 4.6 Hz, Ar-H).

15

¹³C NMR (50 MHz, d₆-DMSO) δ 39.80 (CH₂), 45.47 (N-CH₂), 112.20 (CH), 122.27 (CH), 122.35 (C), 129.86 (CH), 146.95 (C), 149.48 (CH), 149.84 (C), 173.36 (C=O).

Example 23: Synthesis of 2-(4-(pyridin-4-ylmethyleneamino)benzamido)acetic acid (20)

Pyridine-4-carboxaldehyde (0.58 g, 5.41 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, *p*-amino-hippuric acid (1.05 g, 5.41 mmol) and a catalytic amount of TsOH (7 mg) were added and the resulting
20 suspension stirred at room temperature until all the reactants had dissolved. The resulting yellow solution was stirred at room temperature for 12 hours, then concentrated *in vacuo* and placed in the freezer at -20°C. An orange/yellow precipitate formed and was recovered
25 by filtration. The solid was washed with cold methanol and dried *in vacuo* to yield the product (20) as an orange/yellow solid. Yield: >85%.

TLC (MeOH:CH₂Cl₂, 1585)1 major spot, R_f 0.10.

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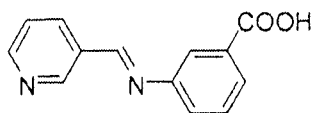
MS $[M+H]^+$ m/z 284.0

1H NMR (200 MHz, d_6 -DMSO) δ 3.96 (2H, m, CH_2), 7.39 (2H, m, Ar-H), 7.60-7.99 (4H, br m, Ar-H), 8.76-8.90 (4H, br m, NH, $CH=N$, Ar-H), 12.87 (1H, br s, COOH).

^{13}C NMR (50 MHz, d_6 -DMSO) 41.21 (CH_2), 121.01 (CH), 122.30(CH), 128.50 (CH),
5 131.86 (C), 142.22(C), 150.47 (CH), 153.14 (C), 160.7 (C=N), 165.86 (C=O), 171.31 (COOH).

Example 24: Synthesis of 3-(pyridin-3-ylmethylenecamino)benzoic acid (21)

Pyridine-3-carboxaldehyde (0.84 g, 7.84 mmol) was dissolved in methanol (*ca.* 30 mL)
10 and the solution stirred at room temperature. To this solution, 3-aminobenzoic acid (1.08 g, 7.84 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within one minute of dissolving, a pale yellow precipitate formed. The suspension was stirred at room temperature for 12 hours at room temperature, filtered, the solid washed with cold
15 methanol and dried *in vacuo* to yield the product (21) as a pale yellow solid. Yield: 98%.



TLC (MeOH: CH_2Cl_2 , 10:90) 1 major spot, R_f 0.15, 0.40 and 0.80.

MS $[M+H]^+$ m/z 227.1

1H NMR (400 MHz, d_6 -DMSO) δ 7.56 (3H, m, Ar-H), 7.85 (2H, m, Ar-H), 8.33 (1H, m, Ar-H), 8.72 (1H, m, Ar-H), 8.78 (1H, s, Ar-H), 9.09 (1H, s, $CH=N$) 13.10 (1H, br s, COOH).

^{13}C NMR (100 MHz, d_6 -DMSO) 121.38 (CH), 124.01 (CH), 125.72 (CH), 127.06 (CH), 129.52 (CH), 131.35 (C), 131.98 (C), 135.08 (CH), 150.54 (CH), 151.19 (C), 152.09 (CH), 159.73 (C=N), 166.97 (COOH).

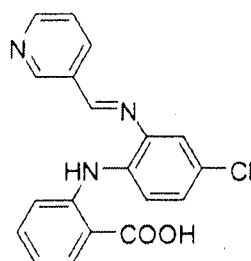
25

Example 25: Synthesis of 2-(4-chloro-2-(pyridin-3-ylmethylenecamino)phenylamino)-benzoic acid (22)

Pyridine-3-carboxaldehyde (0.75 g, 7.00 mmol) was dissolved in methanol (*ca.* 40 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-

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chlorophenyl)-anthranilic acid (1.84 g, 7.00 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a minute of dissolving, a bright orange precipitate formed. The resulting suspension was stirred for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (22) as a bright orange solid. Yield: >95%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.55.

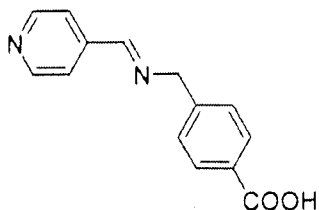
¹H NMR (400 MHz, d₆-DMSO) δ 6.87 (1H, m, Ar-H), 7.27 (1H, m, Ar-H), 7.41-7.59 (5H, br m, Ar-H), 7.94 (1H, m, Ar-H), 8.40 (1H, m, Ar-H), 8.73 (1H, m, Ar-H), 8.88 (1H, s, CH=N), 9.18 (1H, m, Ar-H), 10.49 (1H, br s, NH), 13.21 (1H, br s, COOH).

¹³C NMR (100 MHz, d₆-DMSO) 115.04 (CH), 115.20 (C), 118.18 (CH), 118.54 (CH), 119.05 (CH), 124.51 (CH), 125.37 (C), 127.49 (CH), 132.06 (C), 132.49 (CH), 134.55 (CH), 135.68 (C), 135.85 (CH), 141.06 (C), 145.07 (C), 151.12 (CH), 152.62 (CH), 159.06 (C=N), 170.22 (COOH).

Example 26: Synthesis of 4-((pyridine-4-ylmethyleneamino)methyl)benzoic acid (23)

To pyridine-4-carboxaldehyde (1.05 g, 9.80 mmol) and a catalytic amount of TsOH (10 mg) in a mortar, 4-(aminomethyl)-benzoic acid (0.99 g, 6.54 mmol) was added in portions. After each addition, the reactants were ground together with the mortar and pestle. Upon addition of the final portion of 4-(methyl-amino)-benzoic acid, the mixture was ground to give a pasty white solid, which hardened to a white solid upon standing. This solid was washed with methanol and dried *in vacuo* to yield the product (23) as a white solid. Yield: 90%.

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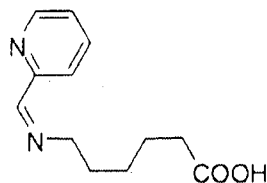
TLC (MeOH:CH₂Cl₂, 10:90) 3 spots, R_f 0.20, 0.25 and 0.40.

¹H NMR (400 MHz, d₆-DMSO) δ 4.91 (2H, s, CH₂), 7.46 (2H, m, Ar-H), 7.73 (2H, m, Ar-H), 7.92 (2H, m, Ar-H), 8.58 (1H, s, CH=N), 8.69 (2H, m, Ar-H), 13.01 (1H, br s, COOH)

5 ¹³C NMR (100 MHz, d₆-DMSO) 64.10 (CH₂), 122.37 (CH), 128.44 (CH), 129.99 (CH), 143.01 (C), 144.62 (C), 147.97 (C), 150.89 (CH), 161.83 (C=N), 167.69 (COOH).

Example 27: Synthesis of 6-(pyridin-2-ylmethyleneamino)hexanoic acid (24)

A mixture of pyridine-2-carboxaldehyde (0.49 g, 4.57 mmol), amino-hexanoic acid (0.60 g, 4.57 mmol) and a catalytic amount of TsOH (7 mg) were added to a 5 mL microwave
10 reaction tube, sealed and heated to 100°C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield a brown oily material, which upon standing solidified to a brown solid. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (24) as a light brown solid. Yield: >95%.



15 ¹H NMR (400 MHz, d₆-DMSO) δ 1.31 (2H, m, CH₂), 1.52-1.65 (4H, m, CH₂), 2.18 (2H, m, CH₂), 3.61 (2H, m, CH₂), 7.45 (1H, m, Ar-H), 7.84-7.94 (2H, m, Ar-H), 8.33 (1H, s, CH=N), 8.63 (1H, m, Ar-H), 12.90 (1H, br s, COOH).

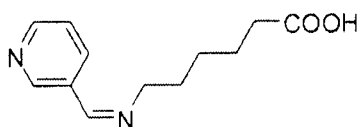
¹³C NMR (100 MHz, d₆-DMSO) 24.90 (CH₂), 26.87 (CH₂), 30.52 (CH₂), 34.33 (CH₂),
20 50.73 (CH₂), 120.87 (CH), 125.51 (CH), 137.33 (CH), 149.82 (CH), 154.71 (C), 162.08 (C=N), 175.01 (COOH).

Example 28: Synthesis of 6-(pyridin-3-ylmethyleneamino)hexanoic acid (25)

A mixture of pyridine-3-carboxaldehyde (0.45 g, 4.20 mmol), amino-hexanoic acid (0.55 g, 4.20 mmol) and a catalytic amount of TsOH (10 mg) were added to a 5 mL microwave
25

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reaction tube, sealed and heated to 100°C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield an orange/brown oily material, which upon standing formed a brown solid. The solid was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (25) as a light brown solid. Yield: >95%.



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¹H NMR (200 MHz, d₆-DMSO) δ 1.27-1.65 (6H, m, CH₂), 2.24 (2H, m, CH₂), 3.57 (2H, m, CH₂), 7.47 (1H, m, Ar-H), 8.09 (1H, m, Ar-H), 8.40 (1H, s, CH=N), 8.62 (1H, m, Ar-H), 8.87 (1H, m, Ar-H), 13.10 (1H, br s, COOH).

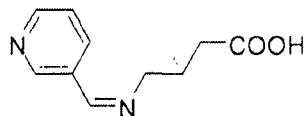
¹³C NMR (50 MHz, d₆-DMSO) 23.81 (CH₂), 25.89 (CH₂), 29.62 (CH₂), 33.23 (CH₂), 60.11 (CH₂), 123.64 (CH), 131.12(C), 133.96 (CH), 149.04 (CH), 150.79 (CH), 157.98 (C=N), 174.05 (COOH).

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Example 29: Synthesis of 4-(pyridin-3-ylmethylenamino)butanoic acid (26)

A mixture of pyridine-3-carboxaldehyde (0.59 g, 5.51 mmol), amino-butanoic acid (0.57 g, 5.51 mmol) and a catalytic amount of TsOH (10 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 100°C for 7.6 minutes. After 7.6 minutes, the reaction vessel was cooled to room temperature to yield an orange/brown solution which upon standing formed a yellow solid. The solid was filtered, washed with cold methanol and dried *in vacuo* to yield the product (26) as a light yellow solid. Yield: 99%.

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¹H NMR (400 MHz, d₆-DMSO) δ 1.81-1.92 (2H, m, CH₂), 2.28 (2H, m, CH₂), 3.59 (2H, m, CH₂), 7.46 (1H, m, Ar-H), 8.11 (1H, m, Ar-H), 8.40 (1H, s, CH=N), 8.62 (1H, m, Ar-H), 8.86 (1H, m, Ar-H), 12.08 (1H, br s, COOH).

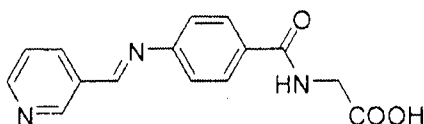
¹³C NMR (100 MHz, d₆-DMSO) 20.56 (CH₂), 26.33 (CH₂), 60.23 (CH₂), 124.39 (CH), 132.00 (C), 134.96 (CH), 150.01 (CH), 151.78 (CH), 159.55 (C=N), 174.73 (COOH).

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Example 30: Synthesis of 2-(4-(pyridin-3-ylmethyleneamino)benzamido)acetic acid (27)

Pyridine-3-carboxaldehyde (0.64 g, 5.98 mmol) was dissolved in methanol (*ca.* 40 mL) and the solution stirred at room temperature. To this solution, *p*-amino-hippuric acid (1.16g, 5.98 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting
5 cream coloured suspension stirred at reflux for 1 hour, then allowed to cool to room temperature and stirred at room temperature for a further 12 hours. The suspension was then filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (27) as a cream coloured solid. Yield: >85%.

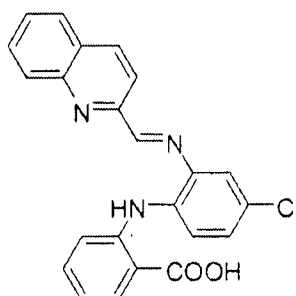


- 10 TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.10
¹H NMR (400 MHz, d₆-DMSO) δ 3.92 (2H, m, CH₂), 7.36 (2H, m, Ar-H), 7.59 (1H, m, Ar-H), 7.96 (2H, m, Ar-H), 8.33 (1H, m, Ar-H), 8.71-8.83 (3H, br m, Ar-H, CH=N), 9.08 (1H, m, NH), 12.56 (1H, br s, COOH).
¹³C NMR (100 MHz, d₆-DMSO) 41.22 (CH₂), 120.88 (CH), 124.06 (CH), 128.46 (C),
15 131.30 (C), 135.13 (CH), 150.57 (CH), 152.21 (CH), 153.63 (C), 159.92 (C=N), 165.86 (C=O), 171.30 (COOH).

Example 31: Synthesis of 2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylamino)-benzoic acid (28)

- 20 Quinoline-2-carboxaldehyde (0.38 g, 2.51 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.66 g, 2.51 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved. Within a minute of dissolving, a bright orange precipitate formed.
25 The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (28) as a bright orange solid. Yield: 95%.

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TLC (MeOH:CH₂Cl₂, 10:90) 3 spots, R_f 0.65, 0.80 and 0.90 (major spot).

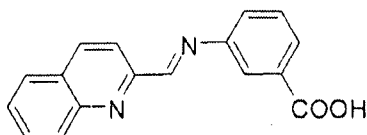
MS [M+H]⁺ m/z 402.3

¹H NMR (200 MHz, d₆-DMSO) δ 6.86 (1H, m, Ar-H), 7.30 (1H, m, Ar-H), 7.49-7.75 (5H, br m, Ar-H), 7.82-7.93 (2H, br m, Ar-H), 8.06-8.016 (2H, br m, Ar-H), 8.49 (2H, m, Ar-H), 8.94 (1H, s, CH=N), 10.53 (1H, br s, NH), 13.21 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 114.39 (CH), 117.38 (CH), 117.88 (CH), 117.98 (CH), 118.32 (CH), 124.58 (C), 126.92 (CH), 127.40 (CH), 127.71 (CH), 128.09 (C), 128.84 (C), 129.92 (CH), 130.69 (CH), 131.56 (CH), 133.60 (CH), 135.15 (C), 136.50 (CH), 139.24 (C), 144.01 (C), 146.94 (C), 154.04 (C), 159.88 (C=N), 169.32 (COOH).

Example 32: Synthesis 3-(quinolin-2-ylmethyleneamino)benzoic acid (29)

A mixture of quinoline-2-carboxaldehyde (0.19 g, 1.20 mmol), 3-aminobenzoic acid (0.17 g, 1.20 mmol), ethanol (3 mL) and a catalytic amount of TsOH (10 mg) were added to a 5 mL mw reaction tube, sealed and heated to 160°C for 3 minutes. After 3 minutes the reaction vessel was cooled to room temperature to yield a brown solution, which upon standing precipitated a brown solid. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (29) as a light brown solid. Yield: 90 %.



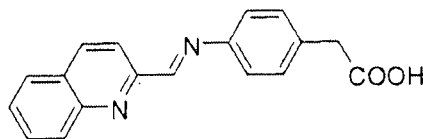
¹H NMR (400 MHz, d₆-DMSO) δ 7.59 (1H, m, Ar-H), 7.66 (2H, m, Ar-H), 7.88 (3H, m, Ar-H), 8.07 (1H, m, Ar-H), 8.14 (1H, m, Ar-H), 8.31 (1H, m, Ar-H), 8.53 (1H, m, Ar-H), 8.81 (1H, s, CH=N) 13.14 (1H, br s, COOH).

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^{13}C NMR (100 MHz, d_6 -DMSO) 118.79 (CH), 122.71 (CH), 125.75 (CH), 128.16 (CH), 128.65 (CH), 129.07 (C), 129.79 (CH), 130.26 (CH), 130.84 (CH), 131.2 (CH), 137.66 (CH) 147.90 (C), 151.04 (C), 154.70 (C), 159.63 (C), 162.47 (C=N), 167.50 (COOH).

5 **Example 33:** Synthesis of 2-(4-(quinolin-2-ylmethyleneamino)phenyl)acetic acid (30)

A mixture of quinoline-2-carboxaldehyde (0.20 g, 1.27 mmol), 4-aminophenylacetic acid (0.19 g, 1.27 mmol), ethanol (3 mL) and a catalytic amount of TsOH (3 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 160 °C for 3 minutes. After 3 minutes the reaction vessel was cooled to room temperature to yield a brown solution. The
10 reaction vessel was placed in the freezer (-20 °C) upon which a brown solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (30) as a brown solid. Yield: >95%.



^1H NMR (400 MHz, d_6 -DMSO) δ 3.63 (2H, m, CH_2), 7.37 (4H, m, Ar-H), 7.69 (1H, m, Ar-H), 7.84 (1H, m, Ar-H), 8.06 (2H, m, Ar-H), 8.29 (1H, m, Ar-H), 8.50 (1H, m, Ar-H), 8.79 (1H, s, CH=N) 12.35 (1H, br s, COOH).

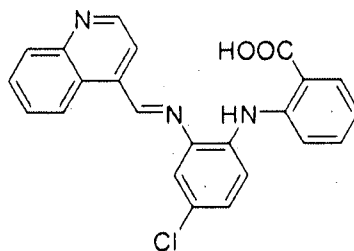
^{13}C NMR (100 MHz, d_6 -DMSO) 40.20 (CH_2), 118.14 (CH), 121.21 (CH), 127.90 (CH), 128.06 (CH), 128.43 (C), 129.18 (CH), 130.21 (CH), 130.35 (CH), 134.08 (C), 136.98 (CH) 147.36 (C), 148.68 (C), 154.41 (C), 160.32 (C=N), 172.55 (COOH).

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Example 34: Synthesis of 2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylamino)benzoic acid (31)

Quinoline-4-carboxaldehyde (0.25 g, 1.59 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. N-(2-amino-4-chlorophenyl)-anthranilic acid
25 (0.42 g, 1.59 mmol) and a catalytic amount of TsOH (3 mg) were then added. The suspension was stirred at room temperature until all reactants had dissolved. Within a minute of dissolving, a red/brown precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (31) as a red solid. Yield: >95%.

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TLC (MeOH:CH₂Cl₂, 10:90) 2 spots, R_f 0.65 and 0.70 (major spot).

MS [M+H]⁺ m/z 402.1

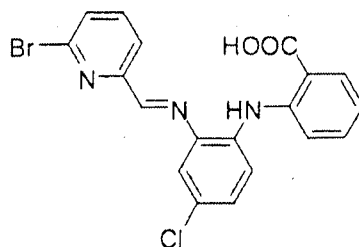
¹H NMR (200 MHz, d₆-DMSO) δ 6.88 (1H, m, Ar-H), 7.33 (1H, m, Ar-H), 7.48 (2H, m, Ar-H), 7.60 (1H, m, Ar-H), 7.72-7.97 (4H, br m, Ar-H), 8.13 (1H, m, Ar-H), 8.24 (1H, m, Ar-H), 9.08 (2H, m, Ar-H), 9.53 (1H, s, CH=N), 10.38 (1H, br s, NH) 13.11 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 112.65 (CH), 118.23 (CH), 118.34 (CH), 119.53 (CH), 120.71 (CH), 123.94 (CH), 124.45 (CH), 124.88 (C), 125.03 (C), 127.23 (CH), 127.30 (C), 131.53 (CH) 133.60 (CH), 134.92 (CH), 137.68 (C), 137.87 (C), 140.69 (C), 144.35 (CH), 148.16 (C), 149.97 (CH), 152.87 (C), 157.94 (C=N), 169.26 (COOH).

Example 35: Synthesis of 2-(2-((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylamino)benzoic acid (32)

6-Bromo-pyridine-2-carboxaldehyde (0.30 g, 1.61 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.43 g, 1.61 mmol) and a catalytic amount of TsOH (3 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved to give an orange solution. Within 15 minutes of dissolving, a red/orange precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (32) as a red solid. Yield: >85%.

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TLC (MeOH:CH₂Cl₂, 10:90) 2 spots, R_f 0.75 and 0.80 (major spot).

MS [M+H]⁺ m/z 430.2

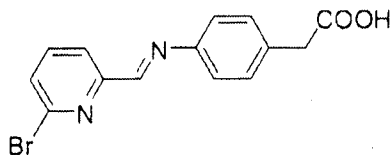
¹H NMR (400 MHz, d₆-DMSO) δ 7.01 (1H, m, Ar-H), 7.30 (1H, m, Ar-H), 7.53-7.89 (8H, br m, Ar-H), 8.09 (1H, m, CH=N), 8.29 (1H, m, NH), 12.64 (1H, br s, COOH).

¹³C NMR (100 MHz, d₆-DMSO) 112.65 (CH), 119.53 (CH), 123.29 (CH), 124.78 (CH), 127.71 (C), 129.10 (CH), 129.65 (CH), 129.88 (CH), 131.86 (CH), 133.69 (CH), 137.18 (C), 137.25 (C), 140.17 (C), 140.78 (CH), 143.43 (C), 149.58 (C), 150.25 (C), 165.44 (C=N), 166.12 (COOH).

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Example 36: Synthesis of 2-(4-((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid (33)

A mixture of 6-bromo-pyridine-2-carboxaldehyde (0.29 g, 1.56 mmol), 4-aminophenylacetic acid (0.24g, 1.56 mmol), ethanol (3 mL) and a catalytic amount of TsOH (3 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150 °C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield a green solution. The reaction vessel was placed in the freezer (-20 °C) upon which a green solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (33) as a green solid. Yield: 95%.



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TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.40.

MS [M+H]⁺ m/z 321.1

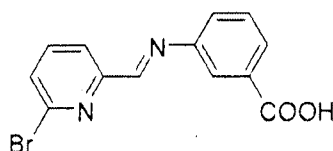
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¹H NMR (200 MHz, d₆-DMSO) δ 3.61 (2H, s, CH₂), 7.32 (4H, m, Ar-H), 7.76 (1H, m, Ar-H), 7.90 (1H, m, Ar-H), 8.15 (1H, m, Ar-H), 8.57 (1H, m, CH=N), 12.22 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 40.74 (CH₂), 120.72 (CH), 121.24 (CH), 129.79 (CH), 130.31 (CH), 134.17 (C), 140.31 (CH), 141.16 (C), 148.36 (C), 155.20 (C), 158.46 (C=N), 172.53 (COOH).

Example 37: Synthesis of 3-((6-bromopyridin-2-yl)methyleneamino)benzoic acid (34)

A mixture of 6-bromo-pyridine-2-carboxaldehyde (0.33 g, 1.77 mmol), 3-aminobenzoic acid (0.24g, 1.77 mmol), ethanol (3 mL) and a catalytic amount of TsOH (3 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150°C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield a dark green solution. The reaction vessel was placed in the freezer (-20°C) upon which a green solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (34) as a green solid. Yield: 97%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, 0.40.

MS [M+H]⁺ m/z 305.1

¹H NMR (200 MHz, d₆-DMSO) δ 7.59 (2H, m, Ar-H), 7.78-7.97 (4H, br m, Ar-H), 8.17 (1H, m, Ar-H), 8.60 (1H, m, CH=N), 13.02 (1H, br s, COOH).

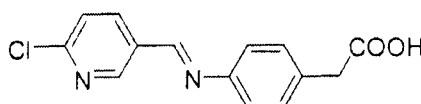
¹³C NMR (50 MHz, d₆-DMSO) 121.16 (CH), 122.13 (CH), 125.36 (CH), 127.70 (CH), 129.69 (CH), 130.11 (CH), 132.06 (C), 140.42 (CH), 141.25 (C), 150.23 (C), 154.93 (C), 160.17 (C=N), 166.88 (COOH).

Example 38: Synthesis of 2-(4-((6-chloromethylpyridin-3-yl)methyleneamino)phenyl)-acetic acid (35)

A mixture of 6-chloro-pyridine-3-carboxaldehyde (0.31 g, 2.19 mmol), 4-aminophenylacetic acid (0.33 g, 2.19 mmol), ethanol (3 mL) and a catalytic amount of

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TsOH (3 mg) were added to a 5 ml microwave reaction tube, sealed and heated to 150 °C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield a yellow solution. The reaction vessel was placed in the freezer (-20 °C) upon which an orange solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (35) as an orange solid. Yield: >85%.



TLC (MeOH:CH₂Cl₂, 10:90) 2 spots, R_f 0.65 and 0.95 (major spot).

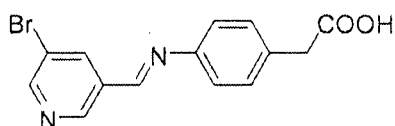
MS [M+H]⁺ m/z 275.1

¹H NMR (200 MHz, d₆-DMSO) δ 3.60 (2H, s, CH₂), 7.24-7.35 (4H, br m, Ar-H), 7.67 (1H, m, Ar-H), 8.34 (1H, m, Ar-H), 8.74 (1H, m, CH=N), 8.89 (1H, m, Ar-H), 12.31 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 46.03 (CH₂), 120.62 (CH), 124.35 (CH), 129.87 (CH), 130.72 (C), 134.86 (C), 137.79 (CH), 148.79 (C), 150.21 (CH), 151.89 (C), 156.48 (C=N), 172.18 (COOH).

Example 39: Synthesis of 2-(4-((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid (36)

A mixture of 5-bromo-pyridine-3-carboxaldehyde (0.35 g, 1.88 mmol), 4-aminophenylacetic acid (0.28g, 1.88 mmol), ethanol (3 mL) and a catalytic amount of TsOH (3 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150°C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield an orange solution. The reaction vessel was placed in the freezer (-20 °C) upon which an orange/yellow solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (36) as a yellow solid. Yield: >85%.



TLC (MeOH:CH₂Cl₂, 10:90) 2 spots, R_f 0.65 and 0.90 (major spot).

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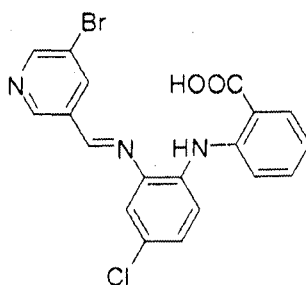
MS $[M+H]^+$ m/z 319.1

1H NMR (200 MHz, d_6 -DMSO) δ 3.61 (2H, s, CH_2), 7.30 (4H, m, Ar-H), 8.50 (1H, m, Ar-H), 8.72 (1H, m, $CH=N$), 8.84 (1H, m, Ar-H), 9.04 (1H, m, Ar-H), 12.30 (1H, br s, COOH).

5 ^{13}C NMR (50 MHz, d_6 -DMSO) 40.15 (CH_2), 120.62 (C), 121.07 (CH), 130.29 (CH), 133.36 (C), 133.80 (C), 137.51 (CH), 148.50 (CH), 149.01 (C), 152.27 (CH) 156.73 (C=N), 172.57 (COOH).

Example 40: Synthesis of 2-((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenyl-amino)benzoic acid (37)

5-bromo-pyridine-3-carboxaldehyde (0.59 g, 3.0 mmol) was dissolved in methanol (*ca.* 30 ml) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.79 g, 3 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved to give an orange solution. Within 1 minute of dissolving, a red/brown precipitate formed. The resulting suspension was stirred for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (37) as an off white solid. Yield: >85%.



20 TLC (MeOH: CH_2Cl_2 10:90) 2 spots, R_f 0.65 and 0.45.

MS $[M+H]^+$ m/z 429.9

1H NMR (200 MHz, d_6 -DMSO) δ 6.89 (1H, m, Ar-II), 7.08 (1H, d, $J = 8.1$ Hz, Ar-II), 7.32 (1H, dd, $J_1 = 8.1$ Hz, $J_2 = 2.0$ Hz, Ar-II), 7.71-7.79 (2H, m, Ar-H), 7.83-7.92 (2H, m, Ar-H), 7.99-8.07 (2H, m, Ar-H, $CH=N$), 8.56 (1H, d, $J = 2.0$ Hz, Ar-H), 8.75 (1H, d, $J = 2.0$ Hz, Ar-H), 9.17 (1H, s, NH).

- 60 -

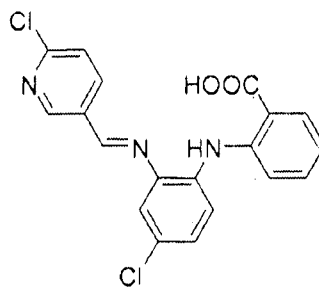
¹³C NMR (50 MHz, d₆-DMSO) 111.83 (CH), 118.98 (CH), 119.71 (C), 124.08 (CH), 127.29 (C), 129.61 (C), 130.08 (CH), 130.41 (CH), 131.73 (CH), 133.93 (CH), 134.23 (C), 136.20 (C), 138.28 (2CH), 143.26 (C), 147.345 (CH), 149.78 (C), 151.11 (C=N), 165.69 (COOH).

5

Example 41: Synthesis of 2-(4-chloro-2-((6-chloropyridin-3-yl)methyleneamino)-phenylamino)benzoic acid (38)

6-Chloro-pyridine-3-carboxaldehyde (0.43 g, 3 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. N-(2-amino-4-chloro-phenyl)-anthranilic acid (0.79 g, 3 mmol) and a catalytic amount of TsOH (10 mg) were added and the resulting suspension stirred at room temperature until all reactants had dissolved to give an orange solution. Within 1 minute, a yellow/orange precipitate formed. The resulting suspension was stirred for a further 12 hours at room temperature, darkening to an orange/red colour, and was then filtered. The solid washed with cold methanol and dried *in vacuo* to yield the product (38) as a dark yellow solid. Yield: >81%.

15



TLC (MeOH:CH₂Cl₂ 10:90) 2 spots, R_f 0.65 and 0.45.

MS [M-H]⁺ m/z 384.1

¹H NMR (200 MHz, d₆-DMSO) δ 7.06 (1H, d, *J* = 8.5 Hz, Ar-H), 7.31 (1H, dd, *J*₁ = 8.5 Hz, *J*₂ = 2.0 Hz, Ar-H), 7.56 (1H, d, *J* = 10.4 Hz, Ar-H), 7.70-7.90 (6H, m, Ar-H), 8.02-8.07 (1H, m, Ar-H), 8.47 (1H, d, *J* = 2.0 Hz, CH=N).

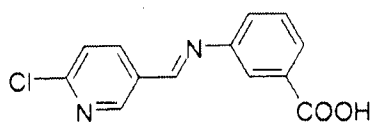
¹³C NMR (50 MHz, d₆-DMSO) 111.76 (CH), 118.90 (CH), 123.93 (CH), 124.25 (CH), 125.19 (C), 127.19 (C), 129.05 (C), 129.63 (CH), 130.04 (CH), 131.78 (CH), 133.95 (CH), 134.31 (C), 136.26 (C), 139.32 (CH), 143.31 (C), 149.37 (CH), 150.24 (C), 159.99 (C=N), 165.71 (COOH).

25

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Example 42: Synthesis of 3-((6-chloropyridin-3-yl)methyleneamino)benzoic acid (39)

6-Chloro-pyridine-3-carboxaldehyde (0.25 g, 1.77 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.24 g, 1.77 mmol) and a catalytic amount of TsOH (3 mg) were added and the resulting suspension stirred at reflux for 12 hours to give a pale yellow suspension. The resulting suspension was then filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (39) as a pale yellow solid. Yield: >85%.



10 TLC (EtOAc:CH₂Cl₂ 1:1) 1 spot, R_f 0.70.

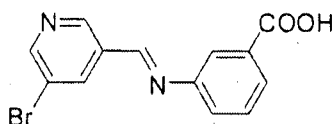
MS [M+H-(COOH)]⁺ m/z 216.0

¹H NMR (400 MHz, d₆-DMSO) δ 7.55 (2H, m, Ar-H), 7.67 (1H, m, Ar-H), 7.85 (2H, m, Ar-H), 8.37 (1H, m, Ar-H), 8.81 (1H, m, CH=N), 8.93 (1H, m, Ar-H), 13.13 (1H, br s, COOH).

15 ¹³C NMR (100 MHz, d₆-DMSO) 121.36 (CH), 124.27 (CH), 125.83 (CH), 127.25 (CH), 129.54 (CH), 130.89 (C), 131.98 (C), 138.32 (CH), 150.79 (CH), 150.87 (C), 152.54 (C), 158.46 (C=N), 166.90 (COOH).

Example 43: Synthesis of 3-((5-bromopyridin-3-yl)methyleneamino)benzoic acid (40)

20 5-Bromo-pyridine-3-carboxaldehyde (0.25 g, 1.34 mmol) was dissolved in methanol (*ca.* 30 ml) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.18 g, 1.34 mmol) and a catalytic amount of TsOH (3 mg) were added and the resulting suspension stirred at reflux for 12 hours to give a pale yellow suspension. The resulting suspension was then filtered, the solid washed with cold
25 methanol and dried *in vacuo* to yield the product (40) as a pale yellow solid. Yield: >95%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.40.

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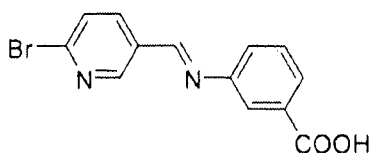
MS $[M+H-(COOH)]^+$ m/z 260.1

1H NMR (200 MHz, d_6 -DMSO) δ 7.57 (2H, m, Ar-H), 7.86 (2H, m, Ar-H), 8.54 (1H, m, Ar-H), 8.79 (1H, m, CH=N), 8.87 (1H, m, Ar-H), 9.08 (1H, m, Ar-H) 13.15 (1H, br s, COOH).

5 ^{13}C NMR (50 MHz, d_6 -DMSO) 120.6 (C), 121.40 (CH), 125.90 (CH), 127.44 (CH), 129.62 (CH), 132.04 (C), 133.15 (C), 137.40 (CH), 148.69 (CH), 150.73 (C) 152.53 (CH), 158.42 (C=N), 166.92 (COOH).

Example 44: Synthesis of 3-((6-bromopyridin-3-yl)methyleneamino)benzoic acid (41)

10 6-Bromo-pyridine-3-carboxaldehyde (0.26 g, 1.40 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.19 g, 1.40 mmol) and a catalytic amount of TsOH (2 mg) were added and the resulting suspension stirred at reflux for 12 hours to give a light brown suspension. The resulting suspension was then filtered, the solid washed with cold
15 methanol and dried *in vacuo* to yield the product (41) as a light brown solid. Yield: 95%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.40.

MS $[M+H-(COOH)]^+$ m/z 260.1

20 1H NMR (200 MHz, d_6 -DMSO) δ 7.56 (2H, m, Ar-H), 7.80-7.88 (3H, m, Ar-H), 8.27 (1H, m, Ar-H), 8.78 (1H, m, CH=N), 8.89 (1H, m, Ar-H), 13.04 (1H, br s, COOH).

^{13}C NMR (50 MHz, d_6 -DMSO) 121.37 (CH), 125.88 (CH), 127.31 (CH), 128.52 (CH), 129.60 (CH), 131.14 (C), 132.00 (C), 137.97 (CH), 144.02 (C), 150.89 (C) 151.23 (CH), 158.69 (C=N), 166.94 (COOH).

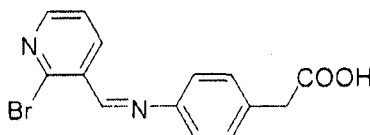
25 **Example 45:** Synthesis of 2-(4-((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid (42)

A mixture of 2-bromo-pyridine-3-carboxaldehyde (0.26 g, 1.40 mmol), 4-aminophenylacetic acid (0.21g, 1.40 mmol), ethanol (3 mL) and a catalytic amount of TsOH (2 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150°C

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for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield an orange solution. The reaction vessel was placed in the freezer (-20°C) upon which an orange/yellow solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (42) as a yellow solid.

5 Yield: >85%.



TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.40.

MS [M-(COOH)]⁺ m/z 273.1

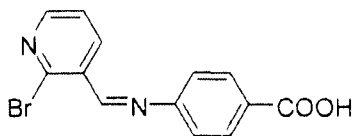
MP 191.1-191.2°C

10 ¹H NMR (200 MHz, d₆-DMSO) δ 3.61 (2H, s, CH₂), 7.29 (4H, m, Ar-H), 7.60 (1H, m, Ar-H), 8.43 (1H, m, Ar-H), 8.53 (1H, m, Ar-H), 8.73 (1H, s, CH=N), 12.57 (1H, br s, COOH).
¹³C NMR (50 MHz, d₆-DMSO) 40.17 (CH₂), 121.02 (CH), 124.07 (CH), 130.40 (CH), 131.53 (C), 134.02 (C), 137.21 (CH), 143.77 (C), 149.18 (C), 152.50 (CH) 157.17 (C=N), 172.55 (COOH).

15

Example 46: Synthesis of 4-((2-bromopyridin-3-yl)methyleneamino)benzoic acid (43)

A mixture of 2-bromo-pyridine-3-carboxaldehyde (0.25 g, 1.34 mmol), 4-aminobenzoic acid (0.18 g, 1.34 mmol), ethanol (3 mL) and a catalytic amount of TsOH (2 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150°C for 5 minutes. After
 20 5 minutes the reaction vessel was cooled to room temperature to yield an orange solution. The reaction vessel was placed in the freezer (-20 °C) upon which an orange/yellow solid precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (43) as a yellow solid. Yield: >95%.



25 TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, R_f 0.40.

MS [M-H]⁺ m/z 305.0

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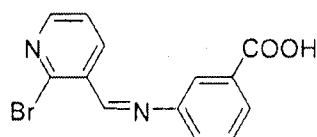
MP 222-223°C

¹H NMR (200 MHz, d₆-DMSO) δ 7.37 (2H, m, Ar-H), 7.63 (1H, m, Ar-H), 8.00 (2H, m, Ar-H), 8.46 (1H, m, Ar-H), 8.56 (1H, m, Ar-H), 8.73 (1H, s, CH=N), 13.11 (1H, br s, COOH).

5 ¹³C NMR (50 MHz, d₆-DMSO) 121.06 (CH), 124.08 (CH), 128.76 (C), 130.67 (CH), 131.21 (C), 137.45 (CH), 143.96 (C), 152.87 (CH), 154.50 (C), 159.30 (C=N), 166.81 (COOH).

Example 47: Synthesis of 3-((2-bromopyridin-3-yl)methyleneamino)benzoic acid (44)

10 A mixture of 2-bromo-pyridine-3-carboxaldehyde (0.27 g, 1.45 mmol), 3-aminobenzoic acid (0.20 g, 1.45 mmol), ethanol (3 mL) and a catalytic amount of TsOH (2 mg) were added to a 5 mL microwave reaction tube, sealed and heated to 150°C for 5 minutes. After 5 minutes the reaction vessel was cooled to room temperature to yield an orange solution. The reaction vessel was placed in a freezer (-20°C) upon which an orange/yellow solid
15 precipitated from solution. The suspension was filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (44) as a yellow solid. Yield: 95%.

TLC (MeOH:CH₂Cl₂, 10:90) 1 major spot, 0.40.MS [M-H]⁺ m/z 303.1

20 MP 203.9-204°C

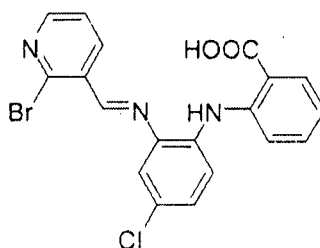
¹H NMR (200 MHz, d₆-DMSO) δ 7.57-7.66 (3H, br m, Ar-H), 7.80-7.91 (2H, m, Ar-H), 8.47 (1H, m, Ar-H), 8.55 (1H, m, Ar-H), 8.76 (1H, s, CH=N), 13.12 (1H, br s, COOH).

¹³C NMR (50 MHz, d₆-DMSO) 121.52 (CH), 124.07 (CH), 125.56 (CH), 127.55 (CH), 129.74 (CH), 131.37 (C), 132.07 (C), 137.40 (CH), 143.89 (C), 150.85 (C), 152.71 (CH),
25 158.75 (C=N), 166.86 (COOH).

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Example 48: Synthesis of 2-((2-((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenyl-amino)benzoic acid: (45)

2-Bromo-pyridine-3-carboxaldehyde (0.23 g, 1.24 mmol) was dissolved in methanol (*ca.* 30 mL) and the solution stirred at room temperature. To this solution, N-(2-amino-4-chlorophenyl)-anthranilic acid (0.32 g, 1.24 mmol) and a catalytic amount of TsOH (2 mg) were added and the resulting suspension stirred at room temperature until all the reactants had dissolved to give an orange solution. Within 15 minutes of dissolving, a yellow precipitate formed. The resulting suspension was left to stir for a further 12 hours at room temperature, filtered, the solid washed with cold methanol and dried *in vacuo* to yield the product (45) as a yellow solid. Yield: >95%.



MS [M-H]⁻ m/z 430.0

M.P 180-180.1°C

¹H NMR (200 MHz, d₆-DMSO) δ 6.89 (1H, m, Ar-H), 7.29 (1H, m, Ar-H), 7.47-7.62 (5H, br m, Ar-H), 7.92 (1H, m, Ar-H), 8.55 (2H, m, Ar-H), 8.84 (1H, s, CH=N), 10.34 (1H, br s, NH) 13.32 (1H, br s, COOH).

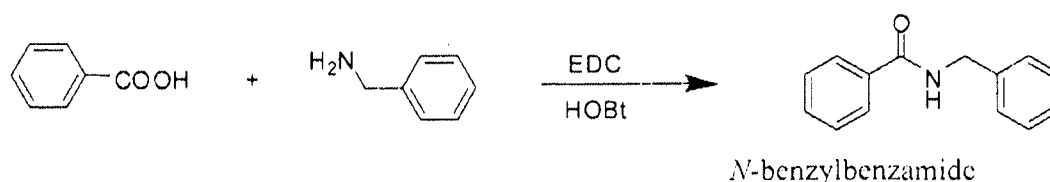
¹³C NMR (50 MHz, d₆-DMSO) 114.69 (CH), 114.78 (C), 117.89 (CH), 118.16 (CH), 118.66 (CH), 123.93 (CH), 124.92 (C), 127.53 (CH), 131.49 (C), 131.89 (CH), 133.99 (CH), 135.09(C), 137.38 (CH), 140.38 (C), 144.00 (C), 144.39 (C), 151.26 (CH), 157.40 (C=N), 169.65 (COOH).

Examples relating to Immobilisation of the Compounds of the Chemical Compound Library

Example 49: Optimisation of the Coupling Conditions for the Immobilisation of Carboxyl-terminated Ligands with Amino-terminated Support Materials

- 5 Optimisation of coupling conditions was initially established using the carboxyl-terminated chemical ligands, e.g. compounds 1-45, with model amines as the mimics of the amino-terminated polymer support materials. The following examples are representative of the procedures employed to select the optimal conditions for this immobilisation.

- (A) Optimisation of coupling conditions using benzoic acid and benzylamine as mimics of
10 the carboxy-terminated ligands and amino-terminated beads with polymer support functionalities respectively.



- Benzoic acid (244.2 mg, 2 mmol) and benzylamine (214.3 mg, 2 mmol) were placed in a
15 reaction vial with the appropriate solvent A, B or C (as detailed below) (10 mL). The contents were stirred and cooled to 0° C and a catalytic amount of triethylamine (TEA, 10 µL) was added. After 5 minutes 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) (460.1 mg, 2.2 mmol) and t-butanol (HOBt) (324.3 mg, 2.2 mmol) were added and the solution was stirred at 0° C for 30 minutes. The ice bath was removed and the solution
20 stirred overnight at room temperature. The solutions were diluted with chloroform or dichloromethane (60 mL) and washed with saturated sodium bicarbonate, 1 M HCl and brine, dried with MgSO₄ and evaporated *in vacuo* to give the crude products. The products obtained from the reactions carried out in dichloromethane (DCM) and MES buffer were isolated immediately whilst the product from the reaction performed in
25 dimethylformamide (DMF) had approximately 1 mL of water added and was subsequently freeze dried. In all cases the final product was a colourless solid.

A: Reaction in DMF: yield 85 %;

B: Reaction in DCM: yield 95 %;

C: Reaction in MES Buffer: yield 92 %.

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The MS and ^1H NMR spectroscopic data for the products were identical in all solvent systems to that below.

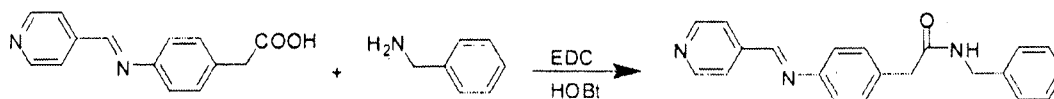
MS $[\text{M}+\text{H}]^+$ m/z 212.0, $[\text{M}+\text{Na}]^+$ m/z 234.0

^1H NMR (300 MHz, CDCl_3) δ 4.65 (2H, d, $J = 6$ Hz, CH_2), 6.66 (1H, bs, NH), 7.28-7.36
 5 (5H, m, Ar-H), 7.40-7.52 (3H, m, Ar-H), 7.79 (2H, dd, $J_1 = 9$ Hz, $J_2 = 2$ Hz, Ar-H).

(B) The above model reaction conditions were then replicated to verify the coupling reaction of the carboxyl-terminated ligands, based on the methyleneamino- and methylamino-heterocyclic structures as indicated above, with benzylamine as the model
 10 for the amino-terminated polymer supports. The following examples are representative of these reaction conditions with these various methyleneamino- and methylamino-heterocyclic ligands.

Example 49A: Coupling of Methyleneamino-Ligands to Amino-group Functionality

15 2-(4-(Pyridin-4-ylmethyleneamino)phenyl)acetic acid (240.2 mg, 1 mmol) and benzylamine (107.2 mg, 1 mmol) were placed in a vial with 10 mL of solvent. The contents were stirred and cooled to 0°C and a catalytic amount of TEA (10 μL) was added. After 5 minutes EDC (210.8 mg, 1.1 mmol) and HOBt (148.6 mg, 1.1 mmol) were added and the solution was stirred at 0°C for 30 minutes. The ice bath was removed and the
 20 solution stirred overnight at room temperature. The solutions were diluted with dichloromethane (60 mL) and washed with saturated sodium bicarbonate, 1 M HCl and brine, dried with MgSO_4 and the organic layer was evaporated *in vacuo* to give the crude product. The product obtained from the reactions carried out in DCM and MES buffer was isolated immediately whilst the product from the reaction performed in DMF had
 25 approximately 1 mL of water added and was subsequently freeze dried. In all cases the final product was a creamy solid.



N-benzyl-2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetamide

Reaction in DMF: yield 65 %;

30 Reaction in DCM: yield 72 %;

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Reaction in MES Buffer: yield 70 %.

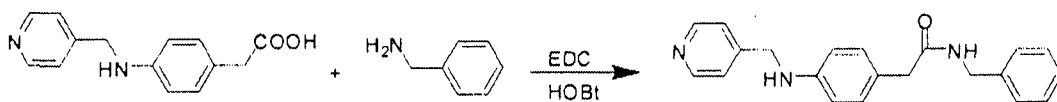
The MS and ^1H NMR spectroscopic data for the product were identical when prepared in all solvent systems as given below.

MS $[\text{M}+\text{H}]^+$ m/z 330.0

- 5 ^1H NMR (200 MHz, $\text{d}_6\text{-DMSO}$) δ 3.52 (2H, s, CH_2), 4.28 (2H, d, CH_2), 7.23-7.38 (9H, m, Ar-H), 7.85 (2H, dd, Ar-H), 8.58 (1H, t, NH), 8.71 (3H, m, Ar-H, $\text{CH}=\text{N}$).

Example 49b: Coupling of Methylamino-Ligands to Amino-group Functionality

- 2-(4-(Pyridin-4-ylmethylamino)phenyl)acetic acid (242.2 mg, 1 mmol) and benzyl-amine
 10 (107.2 mg, 1 mmol) were placed in a vial with the appropriate solvent A, B or C (as detailed below) (10 mL). The contents were stirred and cooled to 0°C and a catalytic amount of TEA (10 μL) was added. After 5 minutes, EDC (210.8 mg, 1.1 mmol) and HOBt (148.6 mg, 1.1 mmol) were added and the solution was stirred at 0°C for 30 minutes. The ice bath was removed and the solution stirred overnight at room temperature.
 15 The solutions were diluted with dichloromethane (60 mL), and washed with saturated sodium bicarbonate, 1M HCl and brine, dried with MgSO_4 and the organic layer was evaporated *in vacuo* to give the crude product. The product obtained from the reactions carried out in DCM and MES buffer was isolated immediately whilst the product from the reaction performed in DMF had approximately 1 mL of water added and was subsequently
 20 freeze dried. In all cases the final product was a creamy solid.



N-benzyl-2-(4-(pyridin-4-ylmethylamino)phenyl)acetamide

Reaction in DMF: yield 73 %;

- 25 Reaction in DCM: yield 81 %;

Reaction in MES Buffer: yield 78 %.

The MS and ^1H NMR spectroscopic data for the product were identical when prepared in all solvent systems as given below.

MS $[\text{M}+\text{H}]^+$ m/z 332.2

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^1H NMR (300 MHz, d_6 -DMSO) δ 3.26 (2H, s, CH_2), 4.22 (2H, d, CH_2), 4.26 (2H, d, CH_2), 6.26 (1H, t, NH), 6.46 (2H, d, Ar-H), 6.95 (2H, d, Ar-H), 7.18-7.33 (7H, m, Ar-H), 8.36 (1H, t, NH), 8.46 (2H, d, Ar-H).

^{13}C NMR (75 MHz, d_6 -DMSO) δ 41.55 (CH_2), 42.07 (CH_2), 45.47 (CH_2), 112.13 (CH), 122.15 (CH), 123.73 (C), 126.58 (CH), 127.08 (CH), 128.11 (CH), 129.44 (CH), 139.50 (C), 146.70 (C), 149.36 (CH), 149.72 (C), 170.77 (C=O).

Example 50: Ligation of Amino-terminated Beads with the Carboxyl-terminated Chemical Ligands based on the above Optimised Procedures:

- Several different coupling conditions were examined based on the above results with the model systems to ligate the carboxyl-terminated ligands to the amino-terminated polymer beads. Initially, a simple literature procedure [Sehgal et al., Analytical Biochemistry, 218 (1994) 87] was employed based on the EDC and NHS coupling method in MES buffer. The procedure was modified accordingly to be consistent with successful and high yielding coupling reactions undertaken in solution phase between acid terminated ligands and the simple model amine (benzylamine) as described above.

The preferred method for ligating the carboxyl-terminated ligands with polymer beads containing terminating amino groups involved performing the reaction in DMF in a similar manner to that observed for the solution phase reactions and is shown in Scheme 4.



Scheme 4

- Initially, the solid support chosen for the ligands was the amino terminated polymer beads (nanospheres), obtained from a commercial supplier (Corpuscular Inc, USA). The beads were used as supplied (50 mg/mL solid concentration, amino group density of 3.3×10^{-6} mol/g, 2 μm average particle size, with a pH 6-7), when stored in deionised water. The carboxyl-terminated ligand (1.65×10^{-8} mol) was made up in DMF (40 μL) and 100 μL of the amino polymer beads (1.65×10^{-8} mol, 1eq) suspended in solution (prepared by

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centrifuging the commercial sample of the amino polymer beads (100 μ L), removing 90 μ L aliquot of the aqueous supernatant and replacing it with 40 μ L of DMF). After 10 minutes of gentle shaking, the coupling reaction was initiated by adding a solution of EDC (1.91×10^{-8} mol) and HOBt (1.81×10^{-8} mol) (1.1 eq. each of EDC, HOBt) in 40 μ L of DMF to the ligand/amino bead solution so that the total volume was 100 μ L. The reaction vials were rotated overnight at room temperature. After the required time, the solutions of reactions 1 and 2 were centrifuged and 90 μ L aliquots were taken out and replaced with deionized water and after shaking the solutions were centrifuged again and 90 μ L aliquots were taken out and replaced with DCM, EtOH and finally water. The ligand coupled beads were stored in water.

Example 51: Optimisation of the Coupling Conditions for Reactions of the Carboxyl-terminated ligands with Hydroxyl terminated support materials

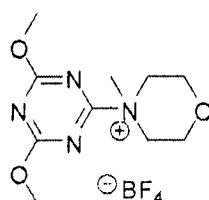
Optimisation of coupling conditions was initially established using carboxyl-terminated ligands with model hydroxyl-group containing compounds as the mimics of the hydroxyl-terminated polymer support materials or hydroxyl-terminated alkylthiol gold surfaces. The following examples are representative of the procedures employed to select the optimal conditions for this immobilization. Several coupling methods, such as CDI, EDC/HOBt/TEA, EDC/HOBt/DMAP, DCC/HOBt/DMAP, were examined to afford the ester bond between the $-\text{COOH}$ terminated ligand and the $-\text{OH}$ terminated support material, but satisfactory coupling product or satisfactory yields could not be obtained routinely with the different carboxyl-group containing ligands. An alternative coupling reaction protocol was thus utilized involving the reagent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium tetrafluoroborate.

Example 52: Synthesis of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium tetrafluoroborate (DMTMM) (46)

N-methyl morpholine (1.10 mL, 10 mmol) was added dropwise to a vigorously stirred solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1.76 g, 10 mmol) in dichloromethane (20 mL) cooled to 5 $^{\circ}\text{C}$. The mixture was stirred at 5 $^{\circ}\text{C}$ for 30 minutes after which time a suspension of silver tetrafluoroborate (1.94 g, 10 mmol) in acetonitrile (20 mL) was added.

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The stirring was continued for an additional 2 hours, the precipitate was filtered off and the filtrate was evaporated to dryness at a temperature not exceeding 20 °C. The solid residue was washed with tetrahydrofuran and recrystallised from acetonitrile/ether to give (46) as a gray solid. Yield 47 %.



5

MS $[M+H]^+$ m/z 241.1

1H NMR (300 MHz, CD_3CN) δ 3.39 (3H, s, CH_3-N), 3.71 (2H, t, $J = 8.5$ Hz, CH_2), 3.75 (2H, t, $J = 10$ Hz, CH_2), 4.00 (2H, m, CH_2), 4.11 (6H, s, O- CH_3), 4.47 (2H, dd, $J_1 = 10$ Hz, $J_2 = 2$ Hz, CH_2).

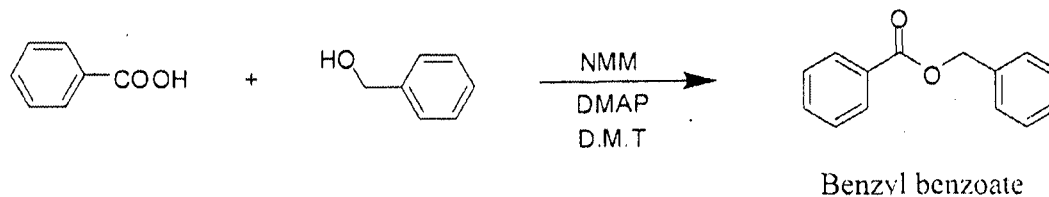
10 ^{13}C NMR (75 MHz, CD_3CN) δ 56.90 (CH_3-N), 57.82 (2 x CH_3-O), 61.10 (2 x CH_2), 62.78 (2 x CH_2), 171.23 (N-C-N), 175.02 (N-C-N).

Example 53:

(A) Optimisation of Coupling Conditions using Benzoic Acid and Benzyl Alcohol as
15 Mimics of the Carboxy-terminated Chemical Ligands and Hydroxyl-terminated Polymer Supported Bead Functionalities respectively.

N-methylmorpholine (68 mg, 0.6 mmol) was added dropwise to a solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium tetrafluoroborate (DMTMM) (200 mg, 0.6 mmol) and benzoic acid 73.3 mg, 0.6 mmol) in acetonitrile (5 mL) at 0 °C. The
20 solution was stirred at 0 °C for an additional 2 hours, then a mixture of benzyl alcohol (81.0 mg, 0.75 mmol) and DMAP (9.3 mg, 0.06 mmol) were added. Stirring was continued at 0° C for 30 minutes and then the mixture was left to stir at room temperature overnight. The solvent was evaporated, and the residue was suspended in chloroform (10 mL). The suspension was washed with water (5 mL), 0.5 M $KHSO_4$ (5 mL), water (5 mL), 0.5 M
25 $NaHCO_3$ (5 mL) and water (5 mL). The organic layer was dried with $MgSO_4$ and the solvent was evaporated *in vacuo* to give the product as a colourless oil. Yield: 85 %

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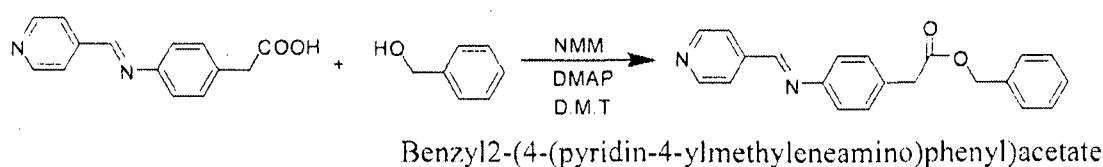
MS $[M+Na]^+$ m/z 225.0

1H NMR (300 MHz, $CDCl_3$) δ 5.43 (2H, s, CH_2), 7.38-7.58 (8H, m, Ar-H), 8.14 (2H, d, J = 8.1 Hz, Ar-H).

(B) The above model reaction conditions were then replicated to verify the coupling reaction of the carboxyl-terminated ligands, based on the methyleneamino- and methylamino-heterocyclic structures as indicated above, with benzyl alcohol as the model
 10 for the hydroxy-terminated polymer bead supports. The following examples are representative of the conditions used in these reactions with these various methyleneamino- and methylamino-heterocyclic ligands.

Example 53a: Coupling of Methyleneamino-Ligands to Hydroxy-group Functionality:

15 *N*-methyl-morpholine (50.6 mg, 0.5 mmol) was added dropwise to a solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium tetrafluoroborate (DMTMM) (164.0 mg, 0.5 mmol) and 2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid (7) (120.1 mg, 0.5 mmol) in acetonitrile (3 mL) at 0 °C. The solution was stirred at 0 °C for an additional 2 hours, then a mixture of benzyl alcohol (67.6 mg, 0.67 mmol) and DMAP
 20 (6.1 mg, 0.05 mmol) were added. Stirring was continued at 0 °C for 30 minutes and then the mixture was left to stir at room temperature overnight. The solvent was evaporated, and the residue was suspended in chloroform (10 mL). The suspension was washed with water (5 mL), 0.5 M $KHSO_4$ (5 mL), water (5 mL), 0.5 M $NaHCO_3$ (5 mL) and water (5 mL). The organic layer was dried with $MgSO_4$ and the solvent was evaporated *in vacuo* to give
 25 the product as colourless oil. Yield: 72 %

MS $[M+H]^+$ m/z 331.1

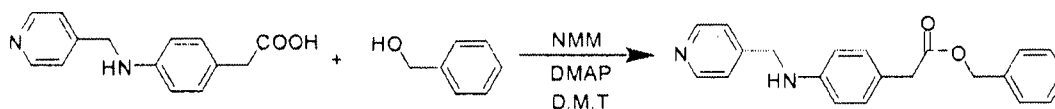
- 73 -

¹H NMR (200 MHz, d₆-DMSO) δ 3.38 (2H, s, CH₂), 4.53 (2H, d, CH₂), 7.20-7.39 (9H, m, Ar-H), 7.84 (2H, dd, Ar-H), 8.67 (1H, s, CH=N), 8.75 (2H, dd, Ar-H).

¹³C NMR (50 MHz, d₆-DMSO) δ 54.76 (CH₂), 62.99 (CH₂), 121.20 (CH), 122.13 (CH), 126.41 (CH), 126.58 (CH), 127.83 (CH), 127.85 (CH), 128.37 (CH), 130.31 (CH), 133.11 (C), 136.01 (C), 142.53 (C), 149.30 (C), 150.41 (CH), 158.90 (CH=N), 170.96 (C=O).

Example 53b: Coupling of Methyleneamino-Ligands to Hydroxy-group Functionality

N-methyl-morpholine (50.6 mg, 0.5 mmol) was added dropwise to a solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium tetrafluoroborate (DMT) (164.0 mg, 0.5 mmol) and 2-(4-(pyridin-4-ylmethylamino)phenyl)acetic acid (7) (121.1 mg, 0.5 mmol) in acetonitrile (3 mL) at 0° C. The solution was stirred at 0 °C for an additional 2 hours, then a mixture of benzyl alcohol (67.6 mg, 0.67 mmol) and DMAP (6.1 mg, 0.05 mmol) were added. Stirring was continued at 0 °C for 30 minutes and then the mixture was left to stir at room temperature overnight. The solvent was evaporated, and the residue was suspended in chloroform (10 mL). The suspension was washed with water (5 mL), 0.5 M KHSO₄ (5 mL), water (5 mL), 0.5 M NaHCO₃ (5 mL) and water (5 mL). The organic layer was dried with MgSO₄ and the solvent was evaporated *in vacuo* to give the product as yellow oil. Yield: 46 %



benzyl 2-(4-(pyridin-4-ylmethylamino)phenyl)acetate

MS [M+H]⁺ m/z 333.1

¹H NMR (200 MHz, d₆-DMSO) δ 3.51 (2H, s, CH₂), 4.30 (2H, d, CH₂), 4.51 (s, BzOH impurity), 5.07 (2H, d, CH₂), 6.33 (2H, d, Ar-H), 6.95 (2H, d, Ar-H), 7.20-7.35 (m, Ar-H, BzOH impurity), 7.84 (2H, dd, Ar-H), 8.47 (2H, d, Ar-H), 8.70 (1H, s, NH), 8.75 (2H, d, Ar-H).

Example 54: Preparation of chemical compound array

Preparation of the Array Silicon Master:

The following example is illustrative of the type of silicon master that can be employed, whereby a PDMS mould can be formed with two series of arrays arranged in two rows,

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each including five arrays of etched 5x5 arrays of pyramidal wells. Other configurational arrangements can be made through changing the location of the pyramidal posts within the silicon master. Figure 1 shows one such fabrication scheme for the preparation of a beads-on-posts microarray. The wells in each row, which have the same size (4.5 or 5.5 μm in width), but placed at an increasing inter-well distance (8, 16, 32, 64 or 128 μm and 6, 12, 24, 48 or 96 μm , respectively), were fabricated by photolithography and anisotropic etching (along the $\langle 100 \rangle$ crystallographic face) of a silicon 4 x 4 inch wafer. The wafer was then cut into smaller chips (about 1 x 1 cm), which were then used for fabrication of the bead-PDMS microarrays. Each chip comprises approximately 60 etched microarrays.

Preparation of Polydimethylsiloxane (PDMS) Polymer

Polydimethylsiloxane (PDMS, Dow Corning) polymer was prepared by mixing the prepolymer base material (184 Silicone Elastomer) and the curing agent (184 Silicone Elastomer) at a ratio of 10:1 (w/w). The mixture was then stirred at room temperature thoroughly until a whitish mixture was present. After degassing for 30 minutes to 1 hour under vacuum until all air bubbles disappeared, the clear and transparent PDMS precursor solution was gently poured over the silicon chip master and cured at 60 – 65 °C for 2 - 3 hours to ensure complete cross-linking. This procedure was employed for the preparation of the PDMS polymer array using the appropriately designed complementary silicon master and array mask. The PDMS replica moulds was then cut and removed from the silicon master and were then ready for deposition of the functionalized beads and further testing and use.

Deposition of the Functionalised Beads into the Etched Wells of the PDMS Mold

Functionalised beads derived from amino-terminated or carboxy-terminated polymeric microspheres, as described below, were deposited inside the etched wells of the PDMS mould formed from the corresponding silicon master, which was prepared as outlined above. The amino-terminated or carboxy-terminated melamine microspheres were obtained from Corpuscular, Inc. (Cold Spring, NY, USA), as a 5 wt.-% aqueous colloidal solution. Amino- or carboxy-terminated beads obtained from other suppliers, such as Estapor-Merck Chimie s.a.s (F-94126 Fontenay Sous Bois Cedex France) or Sigma-

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Aldrich (St. Louis, MO, USA) can also be employed. Ethanol (HPLC grade), acetone (HPLC grade) and DMSO (>99.9%) were purchased from Sigma Aldrich Pty Ltd (Sydney, Australia). All bead solutions were prepared at the approximate concentration of 0.04 wt.-% using MilliQ water. Solutions were always prepared fresh to minimize the formation of
5 aggregates and were vortexed immediately prior to their use for 1-2 minutes.

Functionalised beads can be prepared by immobilising low molecular weight heterocyclic compounds as described in examples 49 to 53. The functionalised beads (with non-functionalised beads lacking the specific ligand(s) employed as controls) were deposited
10 inside the etched wells of the silicon master by spin coating, using a Specialty Coating Systems spin coater (Model P6708). A volume (150 μ L) of the bead solution (0.04 wt.-%), containing either the functionalized or non-functionalised beads, was deposited over the silicon master and the rotor spun to 500 rpm. After 30 seconds, the speed was increased to 2000 rpm and spinning kept for further 60 seconds. Spinning was repeated three times.
15 Between each spin, a thin, flat piece of PDMS was placed over the master to remove the residual beads that deposited on the chip surface outside the wells. The resulting microarray platform thus comprises a transparent, biocompatible poly(dimethylsiloxane (PDMS) polymer moulded into a series of arrayed micro-sized pyramidal posts with single microbeads trapped at their apex. In Figure 2 (a) and (b), close-up microscopic images of
20 the bead trapped in (a) a pyramidal well and (b) at the apex of a PDMS pyramid respectively are illustrated.

The above fabrication method allows the preparation of bead arrays, which are both spatially- and informationally-addressable, in the sense that each bead-array is
25 characterised by a unique geometric code. The trapped beads are mechanically stable and their surface properties are unaltered during the fabrication process, therefore providing anchoring sites for the specific interaction of the immobilised chemical ligands with the target (bio)molecules. Figure 3 shows an example of such a microarray with beads (a) randomly deposited in the wells on the silicon wafer and (b) positioned at the apex of the
30 complementary PDMS pyramids/posts of the microarray.

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When microarrays are intended to be used in conjunction with matrix assisted laser desorption ionisation time-of-flight-mass spectrometry (MALDI TOF MS) instrumentation, which requires spot diameters of larger than 0.5 mm to enable adequate focusing of the MALDI-TOF MS laser, the functionalised beads (with non-functionalised beads lacking the specific ligands employed as controls) can be deposited as chemical compound immobilised bead clusters using, say, 0.5 μL aliquots of approximately 1 mg mL^{-1} bead solution onto the bottom of a preformed polystyrene culture dishes, marked with a 5 x 5 mm² grid on the underside of the dish. In this case, four clusters could then be generated within each 0.5 mm gridbox, resulting in spots of approximately 1 mm in diameter. Figure 4 illustrates the general schematic for the fabrication of these array arrangements. Clearly, other types of bead cluster arrangements can be readily developed, based on the grid design and size. After drying, excess beads were removed with a stream of filtered nitrogen gas, and the PDMS prepolymer poured over the spotted beads to a depth of 3 mm. The PDMS prepolymer was cured as described previously, and any unattached beads and excess buffer salts were removed by extensive rinsing with deionised water. Microscopic visualisation of the bead arrays before and after sonication showed that beads were strongly embedded in the PDMS and could not be removed by sonication at 50 W, 40 kHz. Figure 5 (A)-(D) show examples of this type of bead arrangement so formed, in a 5 x 5 mm grid format, at 20 x and 60 x magnification.

20

Characteristics of the Bead-PDMS Microarray

To test the mechanical stability of the bead-PDMS microarray, the same array was sonicated (separately) in a 20 mL of phosphate buffer saline (PBS, 10 mM), ethanol and acetone for 5, 10 and 20 minutes using an ultrasonic cleaner. Between each wash, the platform was imaged with the 40 x objective of an upright microscope and the number and position of the beads entrapped in the PDMS mould recorded. Depending on the spatial distribution of the functionalised beads, a unique address can be assigned to the specific microarray. For example, Figure 6 illustrates the optical microscopy images of one such array, obtained through the random deposition of the functionalised beads in the wells, reproduced with three different geometric codes.

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Depending on the structure of the immobilised compound, and the type of functionality and chemical composition of the bead itself, different bead-PDMS microarrays, belonging to different chemical compound classes, and derived from different functionalised/non-functionalised beads can thus be obtained. For example, the amino-group reactive dye
5 Alexa Fluor® 546 (Invitrogen, Inc.) was used to test the reactivity of a bead-PDMS microarray fabricated with amino-terminated microspheres (Figure 7). The PDMS stamp was covered with 150 µL of the dye solution (0.4 mg/mL in DMSO) and left reacting for 1 hour. The stamp was then washed with DMSO, water and dried in air. A bead-PDMS microarray fabricated with glass microspheres was also used as a control sample and
10 reacted with the dye Alexa Fluor® 546 under the same conditions. The bead-PDMS microarray when probed with the reactive dye Alexa Fluor® 546 was imaged using an inverted Olympus IX81 microscope (60 x water objective) equipped with a transmitted light differential interference contrast attachment. Fluorescent images (40 x) were captured using the same microscope with epifluorescence optics (Alexa Fluor® 546 filter setting)
15 and mercury light source. The images were recorded with a Coolview FDI high-resolution camera (Photonics Science Ltd.) controlled by Image-Pro Plus software (version 5.0, Media Cybernetics).

When functionalised beads derived from amino- or carboxy-terminated beads are
20 employed, alternative modes of imaging other than fluorescence spectroscopy can be employed, based on the spectral characteristics of the resultant bound analytes, such as polypeptide/peptides generated from the digestion of different target proteins as described below. Moreover, when matrix assisted laser desorption ionisation time-of-flight-mass spectrometry (MALDI TOF MS) is intended to be used as the detection system, depending
25 on the focusing optics of the specific MALDI-TOF MS instrumentation, as noted above microarrays of larger spot dimensions can be employed. For example, with the Voyager DE-STR mass spectrometer manufactured by Applied BioSystems Inc. (Waltham, MA, USA) the optics used for sample visualisation within the MALDI-TOF MS instrumentation are not able to discern beads smaller than 2 µm, so the user would thus not know whether
30 the instrument was sampling an array area with beads or a zone lacking beads, if the spatial distance between beads was of the same dimensions or smaller than the beads themselves.

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A solution to this constraint of focusing the laser of a MALDI-TOF MS instrument can be achieved with the use of beads clustered into spot diameters of approximately 1 mm with spatial displacement between bead arrays of approximately 5 mm. In this manner, regions containing the beads bearing the chemically immobilised ligands can be easily visualised and sampled by the MALDI-TOF MS laser.

SEM Analysis of the PDMS-bead Microarray

SEM analysis of the PDMS-bead microarray was performed on a JEOL 10 microscope (JSM840) at 15 keV. Prior to SEM imaging, the PDMS stamps were coated with gold using Gold coating using a SEM-coating unit E5100 (Polaron Equipment Ltd.) at 25 mA for 90 s at 0.1 Torr.

Example 55: Selection Criteria for the Identification of Specific Peptide/Protein Markers Associated with Specific Bacterial Species:

The following criteria were employed to allow the identification of marker protein/polypeptide/peptides, which are characteristic of various bacterial species and strains:

- ii) the target protein(s) is(are) relatively abundant in bacteria cells;
- iii) the genome sequence and/or proteome of the bacterium was known;
- iv) the expression of the target protein(s) must be essential to cell growth, survival or reproduction, possibly belonging to particular functional group such as cell wall biosynthesis, protein biosynthesis (including the entire tRNA synthetase complex), fatty acid biosynthesis, DNA replication or RNA transcription;
- v) the target protein(s) must have homologues in other target bacteria, but nevertheless is structurally unique;
- vi) the target protein(s) must have sites within its amino acid sequence that are proteolytic accessible;
- vii) the target proteins and some proteolytically-derived fragment(s) thereof must be unique for a particular bacterium and;
- viii) the target proteins and some proteolytically-derived fragment(s) thereof must have specific binding behaviour for individual ligands, which are

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immobilised onto nanosized beads, which can be supported in the array format as described in this application;

- ix) the target proteins or the proteolytically-derived fragment(s) must be detectable in MALDI ToF mass spectrometry.

5

Choice of Bacterial Micro-organism Targets for Biomarker Identification

For the purpose of validating the capabilities of the technology, the following micro-organisms were used as exemplars in investigations associated with the detection of pathogenic bacteria that have been implicated in the disease of dairy cows, namely bovine

10 mastitis;

- a) *Escherichia coli*
- b) *Staphylococcus aureus*
- c) *Streptococcus agalactiae*
- d) *Streptococcus dysgalactiae*
- 15 e) *Streptococcus uberis*

Example 56: Sample Preparation Procedures for the Detection of Specific Markers using the Functionalised Bead Microarray Methods

Lysis of Bacterial Cell Colonies to Obtain the Biomarker Cytoplasmatic Proteins

- 20 Biological samples containing bacterial cells were centrifuged at 4,500 g for 5 minutes at 4 °C to allow a cell pellet to be obtained. The cell pellet can be stored overnight at -20 °C. To achieve lysis, the cell pellet was then resuspended in 1 × PBS (150 mM NaCl [Amresco], 10 mM NaH₂PO₄ buffer [Merck Australia Pty. Ltd, Kilsyth], pH 8.0). On a pro-rata volume basis of 10 mL resuspension solution to 0.25 mL lysis solution, a hen egg white
- 25 lysozyme solution (20 mg/mL) was added to the resuspended pellet preparation. The cell suspension was then sonicated 10 times with 30 second bursts with a one minute delay between sonication bursts. The lysate was recovered by centrifugation at 25,000 g for 30 minutes at 4 °C. The resulting lysate supernatant can be stored at -80 °C as aliquots if necessary.

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For the preparation of bacterial cell lysate supernatants for use in larger scale or control experiments, bacterial colonies were cultured by inoculated into 20 mL 2YT (1.6 % Tryptone [Oxoid]; 1 % yeast extract [Oxoid]; 85 mM NaCl [Amresco]) and grown overnight at 37 °C. Fresh 2YT media (500 mL) was inoculated with 10 mL of this culture
5 and the bacterial colony cultures grown at 37 °C until an OD₆₀₀ of between 0.8 and 1.0 was reached.

Determination of Protein Concentration in Bacterial Samples with the BCA Protein Assay
Bacteria lysate supernatants were diluted 1 in 10, 1 in 50, 1 in 100, 1 in 500 or 1 in 1000
10 with 1 × PBS buffer. For protein standards, solutions of bovine serum albumin (BSA) ranging in concentration from 20 µg/mL to 1000 µg/mL were prepared. Aliquots (100 µL) of the samples and standards were transferred into 2 mL eppendorf tubes. Reagent A and Reagent B from BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL) were mixed together at a ratio of 50:1 (Reagent A : B) and 2 mL of mixture was added to each sample
15 and standard tube. The reactions were incubated at 37 °C for ≤ 30 minutes before the absorbance were measured at 562 nm. Protein concentrations of the samples were then determined from the standard curve.

Ethanol Precipitation of Proteins from Bacterial Cell Lysates

20 Six to eight volume equivalents of cold ethanol (stored overnight at -20 °C) was added to the centrifuged cell lysate. The sample was mixed by vortexing then incubated on ice for 15 minutes and then stored at -20 °C overnight. Proteins were pelleted by centrifuging at 14,000 g for 30 minutes at 4 °C. The pellet was then washed with cold ethanol (4-6 times the starting volume of the centrifuged cell lysate) by vortexing and incubation on ice for 20
25 minutes. The ethanol washed protein was then re-pelleted by centrifugation as above, air-dried and then directly analysed or alternatively could be lyophilized for storage and subsequent analysis.

Enzymatic Digestion of the Precipitated Bacterial Proteins

30 The dried (lyophilized) protein sample was dissolved in 50 mM ammonium bicarbonate (NH₄HCO₃) to give a final concentration of 1 mg/mL. Calcium chloride (CaCl₂) was then

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added to a final concentration of 2 mM. Aliquots (typically ~100 μ L) of the above mixture were incubated at 100 °C for 90 seconds, and then DTT was added to a final concentration of 10 mM. This sample was incubated at 60 °C for 30 minutes, and the sample cooled at room temperature for 30 minutes. For digestion volumes of 100 μ L, 15 μ L of RapiGestTM SF (2 % solution) was added to give a final concentration of ~0.1 %. The digest sample was then vortexed before and after the addition of iodoacetamide to a final concentration of 20 mM, and incubated at room temperature in the dark for 30 minutes. For maximum activity, trypsin was incubated in the digestion buffer at 30 °C for 15 minutes before addition to the sample at a ratio of 1:20 (trypsin:sample). After two hours digestion at 37 °C, the digest was centrifuged at 13,000 x g for 5 minutes at room temperature. The supernatant containing the tryptic digest peptides was then collected before an additional aliquot of trypsin was added at the same ratio and the digestion continued for another 2 hours at 37 °C. The enzyme was inactivated by the addition of TFA (trifluoroacetic acid) to a final concentration of 1-2 % and the sample was centrifuged at 13,000 x g for 5 minutes at room temperature before being concentrated by evaporation. An aliquot (50 μ L) of 0.1 % aqueous formic acid (v/v) was added before the sample was desalted using a C18 OMIX pipette tip (to generate a working volume of ~10 μ L).

Sample Handling and Solid Phase Extraction Methods with Target Polypeptides/Peptides

Prior to sample desalting and concentrating, a C18 OMIX pipette tip was washed three to five times successively with ACN/H₂O (75/25 v/v)/0.1 % TFA followed by 0.1 % TFA three to five times. The sample was then drawn slowly into the pipette tip ten to twenty times. The C18 tip was then washed two to three times with 0.1 % TFA before the sample was eluted in 10 μ L ACN/H₂O (75/25 v/v)/0.1 % TFA by drawing this through the C18 pipette tip two to three times. The tryptic digestion samples (~2 μ g/ μ L) can be dried by evaporation and stored at -20 °C, prior to analysis, if necessary.

Methylation of Phosphopeptides

In order to characterize post-translationally modified proteins/peptides that have been phosphorylated, an additional methylation step is required. In this case, the tryptic digestion sample (containing the phosphopeptides) (2 μ g/ μ L) was reconstituted in methanolic HCl

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(~500 μ L), and methyl esterification was allowed to proceed for 3-4 hours at room temperature. The derived polypeptide/peptides were then recovered by drying using rotary evaporation (~2.7 kPa) at 40 °C, vacuum 50 mbar) before being reconstituted in a solution containing an equal volume of methanol, water, acetonitrile and 0.5 % TFA. The sample
5 was then ready for the micro-array chemical ligand capture and MALDI-TOF analysis. Enzymatically digested β -casein phosphopeptides can be used as internal and external calibration standard.

Dephosphorylation of Phosphopeptides

10 To further characterize post-translationally modified proteins/peptides that have been phosphorylated, a dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) was added to the tryptic digestion sample that had been processed through the C18 OMIX pipette tip (typically in the volume ratio of (4 μ L to 33 μ L). One unit of calf intestinal alkaline phosphatase was then added before the mixture was incubated at 37 °C for one
15 hour. An additional unit of alkaline phosphatase was added after 20 and 40 minutes. The sample was then ready for micro-array chemical ligand capture and MALDI-TOF analysis. Enzymatically dephosphorylated β -casein phosphopeptides can be used as internal and external calibration standard.

20 Solid Phase Extraction with Titanium Oxide Materials for Further Phosphopeptide Enrichment

Prior to phosphopeptides enrichment and concentrating, a TiO MonoTip was washed three times successively with 100 % ACN/0.1 % trifluoroacetic acid followed by the conditioning buffer (200 mM NaH_2PO_4 buffer [Merck Australia Pty. Ltd, Kilsyth], pH 7.0)
25 twice. The sample was then drawn slowly into the pipette tip approximately twenty times. The TiO Monotip was then washed two to seven times with rinsing buffer (50 % ACN with 0.1 % formic acid and 0.1 M KCl) before the sample was eluted in 50 μ L 0.2 M phosphate buffer (pH 7) by drawing this solution through the TiO Monotip three to six times.

Example 57: Techniques Employed for the Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometric (MALDI-TOF MS) Detection Analysis and Characterisation of Biotargets

5 Matrix Preparation Method with 2,5-DHIB (2, 5-dihydroxybenzoic acid, gentisic acid) for MALDI-TOF MS Analysis

2,5-DHB was dissolved in water/acetonitrile (35:65, v/v) and 0.1 % trifluoroacetic acid or 1 % ortho-phosphoric acid to give a final concentration of 10 mg/mL. Solutions of 2, 5-DHB and diammonium citrate (200 mM) were combined at a ratio of 9:1, and vortexed
10 until the matrix was dissolved.

Matrix Preparation Method for THAP (2, 4, 6-trihydroxyacetophenone)

THAP was prepared by dissolving 2, 4, 6-trihydroxyacetophenone monohydrate in water/acetonitrile (50:50, v/v) and 0.1 % TFA or 1 % o-PA to give a final concentration of
15 14 mg/mL. Solutions of THAP and diammonium citrate (200 mM) were combined at a ratio of 9:1, and vortexed until matrix was dissolved.

Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI TOF MS)

20 All protein, polypeptide and peptide products were analyzed with a MALDI ToF mass spectrometer (Voyager DE-STR, Applied BioSystems) with a 337 nm nitrogen laser using 3-nanosecond duration pulses with a maximum firing rate of 20 Hz. Spectra in the reflectron mode were obtained with delayed extraction using a delay time of 375 ns with positive polarity for samples with the 2, 5-DHB as a matrix and 375-550 ns in a positive
25 polarity for samples with the THAP as a matrix. The low mass gate was set at 500 Da. Each spectrum comprised 500 laser shots.

Mass Spectrometry Data Analysis

The MALDI TOF MS spectra were processed with the Data Explorer Software Version
30 4.0.0.0 baseline corrected, noise filtered/smoothed and deisotoped to determine the monoisotopic masses. Spectra were analyzed using the SpectrumMill software where the

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databases were searched with MS spectra obtained for the Peptide Mass Fingerprints (PMF). In this procedure, the IntelliCal routine with one filter was utilized. The pre-processing filter was set to an accuracy of 100 ppm to identify the protein from the obtained masses of the tryptic peptides. The mass spectral interpretation tool for MALDI
5 TOF MS data used was the Peptide Mass Fingerprint data (PMF) subroutine and the database used for the identification of peptides was the non-redundant SwissProt or NCBI database.

Databases for Protein Identification

10 The SpectrumMill workbench allows the use of subset databases for the purpose of fast off-line searches of the results. The databases for the following bacteria (+ strains) were installed on a server and used as off-line searchable databases:

- NCBI nr
- SwissProt
- 15 ➤ *Escherichia coli* (CFT073, K12-MG1655, 0157H7, 0157H7 EDL933)
- *Staphylococcus aureus* (MW2, Mu50, N315, COL, MRSA252, MSSA476)
- *Streptococcus agalactiae* (2603V/R, NEM316, A909)
- *Streptococcus uberis* (0140J)
- *Streptococcus dysgalactiae* (no information: genome not sequenced)

20

Sequence Alignment of Homologous Proteins for Biomarker Identification

In order to identify homologous proteins amongst the bacterial strains (*E. coli*, *Staphylococcus aureus* and *Streptococcus agalactiae*) several protein databases can be utilised. The WU-Blast2 (Washington University Basic Local Alignment Search Tool
25 Version 2.0.) was used due to its high sensitivity as well as the Blastp, UniProt and Blossum62 matrix programs.

Example 58: Application of the New Functionalised Bead-Based Microarray Technology for the Detection, Analysis and Characterisation of Bacterial Peptides

30 Establishment of Protocols for the Evaluation of the Chemical Compounds Immobilised to Beads for their Capacity to Bind Bacterial Target Peptides

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The following protocol is representative of the procedures followed to evaluate the capture efficiency of the embedded beads with immobilised ligands, such as the methyleneamino- and methylamino-heterocyclic compounds 1-45, and to determine their ability to selectively bind bacterial peptides. These binding/capture procedures can be operated in two alternative modes. The first mode, called the positive binding mode, is based on the selective and highly specific binding of target bacterial peptides to the ligands, chemically immobilised onto the beads, which in turn are supported on the posts or other types of polymer surface configurations, generated for example from a PDMS polymer substratum. In this mode the bound bacterial peptides are analysed and characterised directly by MALDI-TOF MS techniques as described below. The second mode, called the negative binding mode, is based on the selective and highly specific binding of target peptides to the ligands, chemically immobilised onto the beads, which in turn are supported on the posts or other types of polymer surface configuration, generated for example from a PDMS polymer substratum. In this mode the non-bound peptides, recovered in the wash fraction, are analysed and characterised directly by MALDI-TOF MS techniques as described below. The specific peptides recognised by the different chemical ligands, such as the methyleneamino- and methylamino-heterocyclic compounds, 1-45, immobilised onto the bead microarray are then characterised by subtraction analysis.

The control-only samples (i.e. also known as the positive control sample since this sample represents bacterial peptides that have not been exposed to any bead or polymer material surfaces) will contain the full complement of detectable bacterial peptides; similarly, in the positive or negative mode the samples derived from exposure of the crude bacterial peptide sample to the unmodified beads (i.e. also known as the negative control sample) are expected to contain those bacterial peptides which are capable of interacting non-specifically with the bead or polymer support substratum; whilst in the positive or negative mode the samples derived from exposure to the chemical compounds immobilised onto the beads (also known as the test samples) should display a depleted peptide profile resulting from the specific interaction of certain peptides with the bead-immobilised ligands. As a consequence, certain peptide *m/z* peaks in the MALDI-TOF mass spectrum should display significant differences in signal intensities with respect to the positive and negative control

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samples. A significant result will be achievable at an appropriate sample dilution where the concentration of peptides is at or just below the total loading capacity of the beads. When the system is operated in the negative binding mode (i.e. the beads are pretreated with crude bacterial peptide samples after loading onto the PDMS array posts) this will equate
5 with an enhanced signal intensity compared to the positive/negative controls; whilst when the system is operated in the positive binding mode (i.e. the beads are pretreated with bacterial peptide samples prior to loading onto the PDMS array posts) this will equate with an depleted signal intensity compared to the positive/negative controls.

10 Characterisation of Bacterial Proteins/Polypeptides/Peptides through their Ability to Selectively bind to Ligands that have been Immobilised onto Functionalised Beads and presented in Bulk or Micro-Array Format

The following example documents the approach followed to characterise the specificity and characteristics of the different immobilised chemical ligands, such as the
15 methyleneamino- and methylamino-heterocyclic compounds 1-45, presented in bulk or micro-array format, to selectively bind to bacterial proteins, polypeptides and peptides. These experiments were carried out in parallel with the untreated bacterial polypeptide/peptide sample (negative control sample), with the unmodified beads (positive control sample) and with the functionalized beads containing different immobilised
20 chemical ligands. Prior to incubation, the unmodified and functionalised bead systems were washed with de-ionized water. Excess liquid was removed by centrifugation of the bead systems and discarded, and the washing procedure repeated. The unmodified and functionalised bead systems were then washed with the equilibration buffer (100 μ L of 10 mM morpholine ethanesulfonic acid, MES, at pH 7.5), and again centrifuged. All steps
25 were performed at room temperature.

Bacterial protein/polypeptide/peptide samples (20 μ L per sample), prepared as described above, were passed twice through C18 OMIX pipette tips. The resulting solutions were evaporated using a high throughput evaporator and the pellets resuspended by gently
30 mixing it for a few minutes in 100 μ L of above equilibration buffer (10 mM morpholine

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ethanesulfonic acid, MES, at pH 7.5). Fifty μL of this solution was then diluted 1:10, 1:100, 1:1000, 1:10000 and 1:100000 $\times$ with the above buffer.

For both the positive and the negative adsorption mode, each bead sample (the unmodified
5 beads and the beads containing immobilised ligands), arranged according to a specific pre-determined order, received aliquots of the protein/polypeptide/peptide solution, prepared as described above, at five different concentrations diluted respectively over a 10^5 range. These samples were then incubated for 5 minutes, briefly washed with incubation buffer and then centrifuged at $13,000 \times g$ for 10 minutes to remove the supernatants and
10 washings. In the case of the positive and negative adsorption mode, both the peptide-bound bead system and the supernatant/washings can be recovered for analysis. An additional control was included which consisted of the original samples of the protein/polypeptide/peptide solutions incubated with buffer only.

15 The general format that can be employed is shown in Figure 8. The chemical ligands, such as the methyleneamino- and methylamino-heterocyclic compounds 1-45, were individually immobilised onto the beads using the procedures described above, whilst the different bead clusters and arrays were made as discussed above by preparing a mould, placing the beads into the indentations in the mould and a solidifiable polymer poured on top then after
20 setting the polymeric array of immobilised beads removed from the mould. The arrangement of the various chemical ligands is operator friendly, i.e. a specific compound, such as 6-(pyridin-2-ylmethyleneamino)hexanoic acid (24), can be located at any position of choice, e.g. at position 11 and/or 12 and/or 13,, and/or 21 and/or 22 and/or 23,,
25 etc, of the XY grid shown in Figure 8 and the other chemical compounds arranged in an ordered rank on adjacent locations according to polarity, hydrophobicity or another physical chemical property, or alternatively randomly. In the preparation of the array format, although it may be feasible to load all available site with beads, technically a 100% occupancy by the functionalised beads may not be desirable or essential. For example, random distribution of the beads on the PDMS polymer surface, prepared as described
30 above, results in groups of arrayed beads, each bearing the same or a different chemical compound, being presented as an informationally-addressable "bar code", which represents

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a quasi-unique signature that can be read separately from the information carried by the beads. All samples were subjected to MALDI TOF MS analysis as triplicate measurements. The positive control sample (i.e. the sample not exposed to either the functionalised beads or the naked beads) contains the full complement of peptides present
5 in the bacterial protein/polypeptides preparation. The product from the negative control sample, when analysed in the positive adsorption mode under sub-optimal washing conditions, is expected to contain those bacterial polypeptides/ peptides that have undergone non-specific binding to the unmodified beads. The product from the incubation of the negative control sample with the unmodified beads under the negative adsorption
10 mode with suboptimal washing conditions will be depleted in those bacterial polypeptide/peptides that have non-specifically bound to beads lacking any immobilised chemical compounds.

Similarly, the product from incubation in the positive adsorption mode with the beads
15 modified with chemical compounds is expected, following an optimised washing step(s), to contain those bacterial polypeptide/peptides that have specifically bound to the beads, whilst in the negative adsorption mode the corresponding wash sample will be depleted in those bacterial polypeptide/peptides that have specifically bound to the bead with immobilised chemical compounds. Thus, in the latter case, it is expected that some
20 polypeptide/peptides will be missing as they interact specifically with certain chemical compounds, such as the methyleneamino- and methylamino-heterocyclic compounds 1-45, immobilised onto the beads. The corresponding MALDI TOF MS m/z signals for these polypeptides/peptides will, as a consequence, be significantly reduced or disappear with respect to the control samples.

25

The following examples, shown as Figures 9 and 10, illustrate the potential of the new technology for micro-organism screening with different chemical compounds immobilised in these bead formats. In this case, the capability is documented with bacterial systems, whereby two closely related compounds, namely compound 7, 4-(pyridine-4-ylmethyleneamino) benzoic acid, and compound 5, 4-(pyridine-3-ylmethyleneamino)benzoic acid, show significantly different binding specificity and
30

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selectivity. As noted above, the positive control sample analyses are carried out with samples not exposed to either the beads or polymer substratum, the negative control sample experiments involve samples exposed to unmodified beads and the test samples involve samples at different dilutions exposed to beads carrying the immobilised chemical
5 ligands, e.g. beads that possess different surface chemical functionalities as immobilised pyridinyl-methyleneamino- or pyridinylmethylamino- related compounds, prepared as described above. Prior to incubation with the sample, the various bead systems were washed and left for about 10 minutes in equilibration buffer. Previously prepared bacterial samples were suspended in the equilibration buffer and serially diluted 10-fold up to a
10 dilution of 10^5 with the equilibration buffer prior to loading onto the various bead systems for analysis. Thus, these bacterial peptide samples were loaded onto the unmodified beads and beads with immobilised ligands, incubated for 5 minutes, and following appropriate wash and other handling procedures as set out above, were then analysed by the above MALDI-TOF MS procedures.

15

In this illustrative example, the MALDI-TOF mass spectral runs for a sample exposed to target capture in the positive adsorption mode is shown in Figures 9 and 10, highlighting the selection of only one m/z value (ions corresponding to other m/z values can also be evaluated as necessary). As evident from these examples, in this case a peptide
20 corresponding to a mass spectral peak value of $m/z = 957.1385$ $[M+H]^+$ is depleted (captured) from the bacterial peptide preparation (Figures 9 and 10, Panel A) by the bead containing the immobilised compound 7, i.e. 4-(pyridine-4-ylmethyleneamino)benzoic acid), but clearly remains on screening the sample with beads that have not been modified with this chemical compound (Figure 9 and 10, Panel B) or in the control peptide sample
25 (Figure 9 and 10, Panel C). Based on database search using the Spectrum Mill software and the archived sequences, this m/z ion of 957.1385 corresponded to the peptide $^{13}\text{LEVVVNER}^{40}$. This peptide is diagnostic of the intracellular DNA-binding protein, Hns, and unequivocally confirms the protein is derived from the bacterial strain, *E.coli* strain K12.

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In contrast, with the bead system that contained the immobilised compound 5, i.e. 4-(pyridine-3-ylmethyleamino)benzoic acid, no depletion (capture) of the bacterial peptide corresponding to a mass spectral peak value of $m/z = 957.1385 [M+H]^+$ was observed. In addition, as apparent from these results, exquisite and unexpected selectivity differences are manifested in these formats by these functionalised beads, bearing different immobilised pyridinylmethyleamino- or pyridinylmethyleamino- related compounds that differ only slightly in terms of their structures.

Independent Validation of the Target Protein, Polypeptide and Peptide Identification by Complementary Liquid Chromatography/Electrospray Ionization Ion-Trap Mass Spectrometry (LC/ESI-MS)

Polypeptide mixtures were separated by gradient elution chromatography performed using an Agilent 1100 Series Capillary HPLC and analysed using an Agilent 1100 Series LC ESI-MS ion Trap SL mass spectrometer. Data analysis was performed using the ChemStation Software (Agilent Technologies HP 1100 Series LC ESI-MS). The eluents employed were A: 0.1% formic acid, in Milli-Q water and B: 0.1 % formic acid in acetonitrile, with a linear gradient from 5-100 % B. The total run time was 185 minutes. Solvents were filtered and degassed by a vacuum degasser. UV detection was employed at 211 nm. The column used was a ZORBAX SB-C18 (0.5 × 150 mm, I.D., 5 µm particle size) with the flow rate set at 4 µL/min and temperature set at 30 °C. The injection volume was typically 2 µL. The experiments were carried out at ambient temperatures (21 ± 1 °C). The electro-spray ionization conditions were: nebulizer 15 psi; dry gas 5.0 L/min; dry temperature 325 °C, the target m/z was $m/z = 791$. The scan range was from 100-1800 m/z ; with positive polarity.

25

LC ESI/MSⁿ spectra were analysed using the Agilent software SpectrumMill version A.03.02. The databank used for the identification of the polypeptides/peptides was the non-redundant NCBI nr database. To increase the accuracy for identification of the polypeptide/peptides other search engines were also used such as the Mascot (databases: NCBI nr and SwissProt) and Phenix (database: NCBI nr). All database searches were performed using the servers MS/MS Ion Search. Detection was based on raw deconvoluted

30

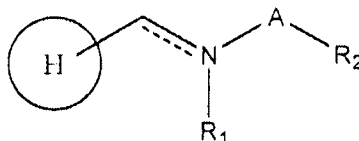
- 91 -

MS data and MS/MS data from the polypeptides/peptides or fragmented peptides. Enzymatically cleaved peptides derived from selected proteins were identified by using the program *Peptide Mass*, [<http://us.expasy.org/>], making allowance for a maximum of 5 missed cleavages. Data analysis and deconvolution of the MS data was performed with the
5 LC ESI-MS Trap Software Version 4.2. This approach enabled the independent validation of the assignment of the target protein as identified by the above described novel technology, and resulted in confirmation that the bacterial strain was *E. coli* K12. This outcome was achieved by complementary LC ESI ion trap mass spectrometry of the tryptic digest of the *E. coli* cell extracts, which again resulted in unambiguous identification of the
10 protein H-ns based on LC ESI/MSⁿ m/z ion profiles at m/z = 957.536 (LEVVVNER), m/z = 1304.564 (YSYVDENGETK), m/z = 1575.725 (REEESAAAAEVEER) and m/z = 2226.169 (EMLIADGIDPNELLNSLA AVK). The identification of these m/z ions, and the retrieval of the corresponding peptides by this technique provides the independent validation for the efficacy of the immobilised chemical ligands, attached to beads, to
15 screen for and detect bacterial micro-organisms, such as those associated with bovine mastitis.

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CLAIMS

1. A chemical library comprising at least two compounds of formula (I):



(I)

wherein

H is an optionally substituted nitrogen containing heteroaryl group;

10 ----- is selected from a single and double bond;

R₁ is hydrogen when ----- is a single bond and absent when ----- is a double bond;

A is a divalent linker;

R₂ is a functional group suitable for attachment to a solid support.

- 15 2. A chemical library according to claim 1, wherein H is selected from pyrrolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,3-triazinyl, indolyl, indazolyl, quinolinyl, isoquinolinyl, cinnolinyl, quinazolinyl, quinoxalinyl, 1,8-naphthyridinyl, 1,7-naphthyridinyl, 1,6-naphthyridinyl, 1,5-naphthyridinyl, phthalazinyl, benzo-1,2,3-triazinyl, benzo-1,2,4-triazinyl, oxazole, 20 thiazole, benzoxazole, benzothiazole and pteridinyl, each of which is optionally substituted.

3. A chemical compound library according to claim 2, wherein H is pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,2,3-triazinyl, 1,2,4-triazinyl, 1,3,5-triazinyl, 25 quinolinyl and cinnolinyl, each of which is optionally substituted.

4. A chemical compound library according to claim 3, wherein H is pyridinyl and quinolinyl, each of which is optionally substituted.

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5. A chemical compound library according to any one of claims 1 to 4, wherein the divalent linker is selected from $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted.

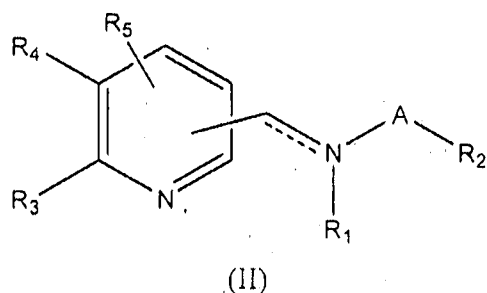
6. A chemical compound library according to claim 5, wherein the divalent linker group is selected from $-(CH_2)_{1-6}-$, $-aryl-$, $-(CH_2)_{1-3}-aryl-$, $-(CH_2)_{1-3}-aryl-(CH_2)_{1-3}-$, $-aryl-(CH_2)_{1-3}-$, $-aryl-NH-(CH_2)_{1-3}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-3}-$, $-aryl-CONH-aryl-$, wherein each aryl is optionally substituted.

7. A chemical compound library according to claim 6, wherein the divalent linker is selected from $-(CH_2)_{1-6}-$, $-phenyl-$, $-(CH_2)_{1-3}-phenyl-$, $-phenyl-(CH_2)_{1-3}-$, $-(CH_2)_{1-3}-phenyl-(CH_2)_{1-3}-$, $-phenyl-NH-(CH_2)_{1-3}-$, $-phenyl-NH-phenyl-$, $-phenyl-CONH-(CH_2)_{1-3}-$ and $-phenyl-CONH-phenyl$, wherein each phenyl is optionally substituted.

8. A chemical compound library according to claim 7, wherein the divalent linker is selected from $-(CH_2)_{1-6}-$, $-phenyl-$, $-CH_2-phenyl-$, $-phenyl-CH_2-$, $-phenyl-NH-phenyl$ and $-phenyl-CONH-CH_2-$ wherein each phenyl is optionally substituted.

9. A chemical compound library according to any one of claims 1 to 8, wherein R_2 is selected from CO_2H , $-NH_2$ and $-OH$.

10. A chemical library according to any one of claims 1 to 9, comprising at least two compounds of formula (II):



wherein

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----- is a double or single bond;

R₁ is hydrogen, when ----- is a single bond and absent when ----- is a double bond;

A is a divalent linker;

R₂ is a functional group suitable for attachment to a solid support;

- 5 R₃ to R₅ are each independently selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl), -NH(C₁₋₆alkyl)₂ and -(OCH₂CH₂)_nCH₂OH wherein n is 1 to 30; or R₃ and R₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted with one or more R₅.

10

11. A chemical compound library according to any one of claims 1 to 10, comprising two or more compounds selected from:

- 2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid,
 2-(4-(pyridin-2-ylmethylamino)phenyl)acetic acid,
 15 2-(pyridin-2-methyleneamino)benzoic acid,
 4-((pyridin-2-ylmethyleneamino)methyl)benzoic acid,
 4-(pyridin-3-ylmethyleneamino)benzoic acid,
 4-(pyridin-3-ylmethylamino)benzoic acid,
 4-(pyridin-4-ylmethyleneamino)benzoic acid,
 20 4-(pyridin-4-ylmethylamino)benzoic acid,
 4-(pyridin-2-ylmethyleneamino)benzoic acid,
 4-(pyridin-2-ylmethylamino)benzoic acid,
 3-(pyridin-2-ylmethyleneamino)benzoic acid,
 3-(pyridin-4-ylmethyleneamino)benzoic acid,
 25 2-(4-chloro-2-(pyridin-4-ylmethyleneamino)phenylamino)benzoic acid,
 2-(4-chloro-2-(pyridin-2-ylmethyleneamino)phenylamino)benzoic acid,
 2-(4-(pyridin-3-ylmethyleneamino)phenyl)acetic acid,
 2-(4-(pyridin-3-ylmethylamino)phenyl)acetic acid,
 2-(pyridin-4-ylmethyleneamino)benzoic acid,
 30 2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid,
 2-(4-(pyridin-4-ylmethylamino)phenyl)acetic acid,

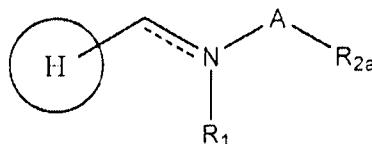
- 95 -

- 2-(4-(pyridin-4-ylmethyleneamino)benzamido)acetic acid,
 3-(pyridin-3-ylmethyleneamino)benzoic acid,
 2-(4-chloro-2-(pyridin-3-ylmethyleneamino)phenylamino)benzoic acid,
 4-((pyridin-4-ylmethyleneamino)methyl)benzoic acid,
 5 6-(pyridin-2-methyleneamino)hexanoic acid,
 6-(pyridin-3-ylmethyleneamino)hexanoic acid,
 4-(pyridin-3-ylmethylenamino)butanoic acid,
 2-(4-(pyridin-3-ylmethyleneamino)benzamido)acetic acid,
 2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylamino)benzoic acid,
 10 3-(quinolin-2-ylmethyleneamino)benzoic acid,
 2-(4-(quinolin-2-ylmethyleneamino)phenyl)acetic acid,
 2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylamino)benzoic acid,
 2-(2-(((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
 2-(4-(((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid,
 15 3-(((6-bromopyridin-2-yl)methyleneamino)benzoic acid,
 2-(4-(((6-chloropyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(4-(((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 2-(2-(((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
 2-(4-chloro-2-(((6-chloropyridin-3-yl)methyleneamino)phenylamino)benzoic acid,
 20 3-(((6-chloropyridin-3-yl)methyleneamino)benzoic acid,
 3-(((5-bromopyridin-3-yl)methyleneamino)benzoic acid,
 3-(((6-bromopyridin-3-yl)methyleneamino)benzoic acid,
 2-(4-(((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 4-(((2-bromopyridin-3-yl)methyleneamino)benzoic acid,
 25 3-(((2-bromopyridin-3-yl)methyleneamino)benzoic acid, and
 2-(2-(((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid.

12. A chemical compound array comprising

- a) a functionalised solid support; and
 30 b) at least one compound of formula (IA):

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(IA)

wherein

5 H is an optionally substituted nitrogen containing heteroaryl group;

----- is selected from a single and double bond;

R₁ is hydrogen when ----- is a single bond and absent when ----- is a double bond;

A is a divalent linker;

R_{2a} represents a functional group attached to the functionalised solid support.

10

13. A chemical compound array according to claim 12, wherein H is selected from pyrrolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,2,4-triazinyl, 1,2,3-triazinyl, indolyl, indazolyl, quinolinyl, isoquinolinyl, cinnolinyl, quinazolinyl, quinoxaliny, 1,8-naphthyridinyl, 1,7-naphthyridinyl, 1,6-naphthyridinyl, 1,5-naphthyridinyl, phthalazinyl, benzo-1,2,3-triazinyl, benzo-1,2,4-triazinyl, oxazole, 15 thiazole, benzoxazole, benzothiazole and pteridinyl, each of which is optionally substituted.

14. A chemical compound array according to claim 13, wherein H is pyridinyl, 20 pyridazinyl, pyrimidinyl, pyrazinyl, 1,2,3-triazinyl, 1,2,4-triazinyl, 1,3,5-triazinyl, quinolinyl and cinnolinyl, each of which is optionally substituted.

15. A chemical compound array according to claim 14, wherein H is pyridinyl and quinolinyl, each of which is optionally substituted.

25

16. A chemical compound array according to any one of claims 12 to 15, wherein the divalent linker is selected from -(CH₂)₁₋₁₀-, -aryl-, -(CH₂)₁₋₅-aryl-, -(CH₂)₁₋₅-aryl-(CH₂)₁₋₅-, -aryl-(CH₂)₁₋₅-, -aryl-NH-(CH₂)₁₋₅-, -aryl-NH-aryl-, -aryl-CONH-(CH₂)₁₋₅-, -aryl-CONH-aryl-, -aryl-CO-(CH₂)₁₋₅-, and -aryl-CO-aryl-, where each aryl is optionally substituted.

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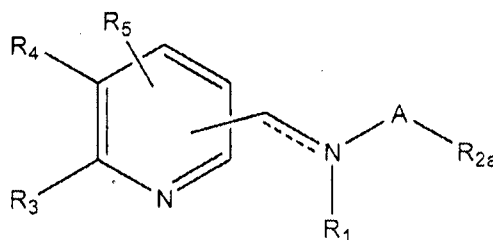
17. A chemical compound array according to claim 16, wherein the divalent linker is selected from $-(CH_2)_{1-6}-$, $-aryl-$, $-(CH_2)_{1-3}-aryl$, $-(CH_2)_{1-3}-aryl-(CH_2)_{1-3}-$, $-aryl-(CH_2)_{1-3}-$, $-aryl-NH-(CH_2)_{1-3}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-3}-$, $-aryl-CONH-aryl-$, wherein
 5 each aryl is optionally substituted.

18. A chemical compound array according to claim 17, wherein the divalent linker is selected from $-(CH_2)_{1-6}-$, $-phenyl-$, $-(CH_2)_{1-3}-phenyl-$, $-phenyl-(CH_2)_{1-3}-$, $-(CH_2)_{1-3}-phenyl-(CH_2)_{1-3}-$, $-phenyl-NH-(CH_2)_{1-3}-$, $-phenyl-NH-phenyl-$, $-phenyl-CONH-(CH_2)_{1-3}-$ and
 10 $-phenyl-CONH-phenyl$, wherein each phenyl is optionally substituted.

19. A chemical compound array according to claim 18, wherein the divalent linker is selected from $-(CH_2)_{1-6}-$, $-phenyl-$, $-CH_2-phenyl-$, $-phenyl-CH_2-$, $-phenyl-NH-phenyl$ and $-phenyl-CONH-CH_2-$ wherein each phenyl is optionally substituted.

15

20. A chemical compound array according to claim 12 comprising
 a) a functionalised solid support; and
 b) at least one compound of formula (IIA):



20

(IIA)

wherein

----- is a double or single bond;

R₁ is hydrogen, when ----- is a single bond and absent when ----- is a double bond;

25 A is a divalent linker;

R_{2a} represents a functional group attached to the functionalised solid support;

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R₃ to R₅ are each independently selected from hydrogen, -C₁₋₆alkyl, -C₂₋₆alkenyl, -halo, -nitro, -OH, -OC₁₋₆alkyl, -SH, -Salkyl, -CN, -NH₂, -NH(C₁₋₆alkyl), -NH(C₁₋₆alkyl)₂ and -(OCH₂CH₂)_nCH₂OH wherein n is 1 to 30, or R₃ and R₄ taken together with the carbon atoms to which they are attached form a 6 membered aromatic ring optionally substituted
5 with one or more R₅

21. A chemical compound array according to any one of claims 12 to 20, comprising one or more compounds selected from:

- 2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid,
- 10 2-(4-(pyridin-2-ylmethylamino)phenyl)acetic acid,
- 2-(pyridin-2-methyleneamino)benzoic acid,
- 4-((pyridin-2-ylmethyleneamino)methyl)benzoic acid,
- 4-(pyridin-3-ylmethyleneamino)benzoic acid,
- 4-(pyridin-3-ylmethylamino)benzoic acid,
- 15 4-(pyridin-4-ylmethyleneamino)benzoic acid,
- 4-(pyridin-4-ylmethylamino)benzoic acid,
- 4-(pyridin-2-ylmethyleneamino)benzoic acid,
- 4-(pyridin-2-ylmethylamino)benzoic acid,
- 3-(pyridin-2-ylmethyleneamino)benzoic acid,
- 20 3-(pyridin-4-ylmethyleneamino)benzoic acid,
- 2-(4-chloro-2-(pyridin-4-ylmethyleneamino)phenylamino)benzoic acid,
- 2-(4-chloro-2-(pyridin-2-ylmethyleneamino)phenylamino)benzoic acid,
- 2-(4-(pyridin-3-ylmethyleneamino)phenyl)acetic acid,
- 2-(4-(pyridin-3-ylmethylamino)phenyl)acetic acid,
- 25 2-(pyridin-4-ylmethyleneamino)benzoic acid,
- 2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid,
- 2-(4-(pyridin-4-ylmethylamino)phenyl)acetic acid,
- 2-(4-(pyridin-4-ylmethyleneamino)benzamido)acetic acid,
- 3-(pyridin-3-ylmethyleneamino)benzoic acid,
- 30 2-(4-chloro-2-(pyridin-3-ylmethyleneamino)phenylamino)benzoic acid,
- 4-((pyridin-4-ylmethyleneamino)methyl)benzoic acid,

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- 6-(pyridin-2-methyleneamino)hexanoic acid,
- 6-(pyridin-3-ylmethyleneamino)hexanoic acid,
- 4-(pyridin-3-ylmethylenamino)butanoic acid,
- 2-(4-(pyridin-3-ylmethyleneamino)benzamido)acetic acid,
- 5 2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylamino)benzoic acid,
- 3-(quinolin-2-ylmethyleneamino)benzoic acid,
- 2-(4-(quinolin-2-ylmethyleneamino)phenyl)acetic acid,
- 2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylamino)benzoic acid,
- 2-(2-(((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
- 10 2-(4-(((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid,
- 3-(((6-bromopyridin-2-yl)methyleneamino)benzoic acid,
- 2-(4-(((6-chloropyridin-3-yl)methyleneamino)phenyl)acetic acid,
- 2-(4-(((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
- 2-(2-(((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
- 15 2-(4-chloro-2-(((6-chloropyridin-3-yl)methyleneamino)phenylamino)benzoic acid,
- 3-(((6-chloropyridin-3-yl)methyleneamino)benzoic acid,
- 3-(((5-bromopyridin-3-yl)methyleneamino)benzoic acid,
- 3-(((6-bromopyridin-3-yl)methyleneamino)benzoic acid,
- 2-(4-(((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
- 20 4-(((2-bromopyridin-3-yl)methyleneamino)benzoic acid,
- 3-(((2-bromopyridin-3-yl)methyleneamino)benzoic acid, and
- 2-(2-(((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid.

22. An assay for detecting the presence of one or more bacteria in a test sample
 25 comprising:

- i) contacting a chemical compound array according to any one of claims 12 to 21, with a test sample that is suspected of containing a bacterial peptide or protein that interacts with at least one chemical compound in the chemical compound array; and
- (ii) detecting the interaction between the bacterial peptide or protein and the at
 30 least one chemical compound in the array.

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23. An assay according to claim 22, wherein the chemical compound array comprises at least one compound of formula IIA as defined in claim 20.

24. An assay according to claim 22 or claim 23, wherein the bacterial peptide is an
5 *E. coli* bacterial peptide.

25. An assay according to claim 24, wherein the bacterial peptide is selected from at least one of:

LEVVVNER [SEQ ID NO: 1]

10 YSYVDENGETK [SEQ ID NO: 2]

RFEESAAAAEVEER [SEQ ID NO: 3] and

EMLIADGIDDNELLNSLA AVK [SEQ ID NO: 4].

26. A method of detecting bacteria in a test sample comprising:

15 i) providing a chemical compound array according to any one of claims 12 to 21;

ii) contacting the array with a biological test sample of interest,

v) determining any interaction of a bacterial peptide marker in the test sample with a chemical compound in the chemical compound array, and

20 vi) identifying the bacterial peptide marker and the bacterium from which the peptide marker is derived.

27. A method of detecting a bacterium responsible for bovine mastitis comprising:

i) providing a chemical compound array according to claim 20;

25 ii) contacting the array with a bovine test sample of interest,

vi) determining any interaction of a bacterial peptide marker in the test sample with a chemical compound in the chemical compound array, and

vii) identifying the bacterial peptide marker and the bacterium from which the peptide marker is derived.

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28. A method according to claim 27, wherein the bacterial peptide marker is selected from at least one of:

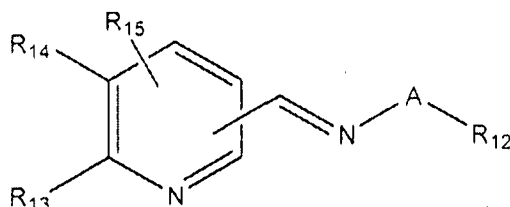
LEV VVNER [SEQ ID NO: 1]

YSYVDENGETK [SEQ ID NO: 2]

5 REEF SAAAAEVEER [SEQ ID NO: 3] and

EMLIADGIDDNELLNSLA AVK [SEQ ID NO: 4].

29. A compound having the formula (III)



(III)

wherein

A is a divalent linker selected from the group $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted;

R₁₂ is selected from $-CO_2H$, $-NH_2$ and OH ;

R₁₃ and R₁₄ are independently selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$; or R₁₃ and R₁₄ taken together with the carbon atoms to which they are attached form a 6

membered aromatic ring optionally substituted with one or more R₁₅; and

R₁₅ is selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$;

with the proviso that the following are excluded:

2-(pyridin-2-methyleneamino)benzoic acid,

4-(pyridin-3-ylmethyleneamino)benzoic acid,

4-(pyridin-4-ylmethyleneamino)benzoic acid,

4-(pyridin-2-ylmethyleneamino)benzoic acid,

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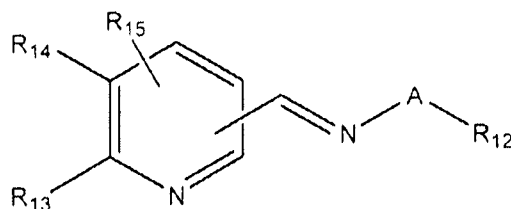
- 3-(pyridin-2-ylmethyleneamino)benzoic acid,
3-(pyridin-4-ylmethyleneamino)benzoic acid,
2-(pyridin-4-ylmethyleneamino)benzoic acid,
2-(4-(pyridin-4-ylmethyleneamino)phenyl)acetic acid,
5 3-(pyridin-3-ylmethyleneamino)benzoic acid, and
2-(4-(quinolin-2-ylmethyleneamino)phenyl)acetic acid.

30. A compound selected from the group consisting of:
2-(4-(pyridin-2-ylmethyleneamino)phenyl)acetic acid,
10 4-((pyridin-2-ylmethyleneamino)methyl)benzoic acid,
2-(4-chloro-2-(pyridin-4-ylmethyleneamino)phenylamino)benzoic acid,
2-(4-chloro-2-(pyridin-2-ylmethyleneamino)phenylamino)benzoic acid,
2-(4-(pyridin-3-ylmethyleneamino)phenyl)acetic acid,
2-(4-(pyridin-4-ylmethyleneamino)benzamido)acetic acid,
15 2-(4-chloro-2-(pyridin-3-ylmethyleneamino)phenylamino)benzoic acid,
4-((pyridin-4-ylmethyleneamino)methyl)benzoic acid,
6-(pyridin-2-ylmethyleneamino)hexanoic acid,
6-(pyridin-3-ylmethyleneamino)hexanoic acid,
4-(pyridin-3-ylmethyleneamino)butanoic acid,
20 2-(4-(pyridin-3-ylmethyleneamino)benzamido)acetic acid,
2-(4-chloro-2-(quinolin-2-ylmethyleneamino)phenylamino)benzoic acid,
3-(quinolin-2-ylmethyleneamino)benzoic acid,
2-(4-chloro-2-(quinolin-4-ylmethyleneamino)phenylamino)benzoic acid,
2-(2-((6-bromopyridin-2-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
25 2-(4-((6-bromopyridin-2-yl)methyleneamino)phenyl)acetic acid,
3-((6-bromopyridin-2-yl)methyleneamino)benzoic acid,
2-(4-((6-chloropyridin-3-yl)methyleneamino)phenyl)acetic acid,
2-(4-((5-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
2-(2-((5-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid,
30 2-(4-chloro-2-((6-chloropyridin-3-yl)methyleneamino)phenylamino)benzoic acid,
3-((6-chloropyridin-3-yl)methyleneamino)benzoic acid,

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- 3-((5-bromopyridin-3-yl)methyleneamino)benzoic acid,
 3-((6-bromopyridin-3-yl)methyleneamino)benzoic acid,
 2-(4-((2-bromopyridin-3-yl)methyleneamino)phenyl)acetic acid,
 4-((2-bromopyridin-3-yl)methyleneamino)benzoic acid,
 5 3-((2-bromopyridin-3-yl)methyleneamino)benzoic acid, and
 2-(2-((2-bromopyridin-3-yl)methyleneamino)-4-chlorophenylamino)benzoic acid.

31. A method of preparing a compound of formula (IV)



(IV)

wherein

A is a divalent linker selected from the group $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted;

R₁₂ is selected from $-CO_2H$, $-NH_2$ and OH;

R₁₃ and R₁₄ are independently selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$; or R₁₃ and R₁₄ taken together with the carbon atoms to which they are attached form a 6

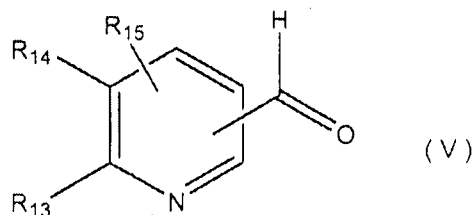
membered aromatic ring optionally substituted with one or more R₁₅; and

R₁₅ is selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$,

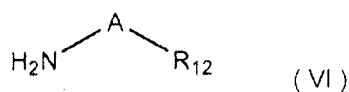
said method comprising

- x) grinding a carboxaldehyde of formula (V)

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wherein R_{13} to R_{15} are as defined in formula (IV)
together with an amine compound of formula (VI)

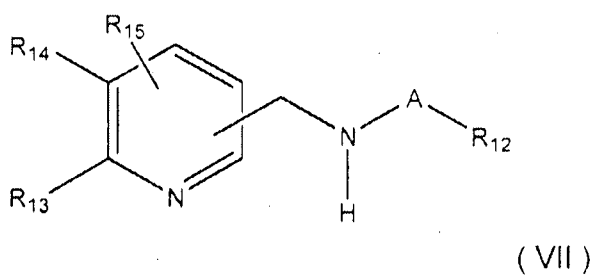


wherein A and R_{12} are as defined in formula (IV)
together with a catalytic amount of an organic acid,

- ii) adding solvent in an amount sufficient to form a paste,
- iii) grinding the paste for a time sufficient for reaction to occur, and
- iv) removing the solvent to provide a compound of formula (IV).

32. A method according to claim 31 further comprising the steps of:

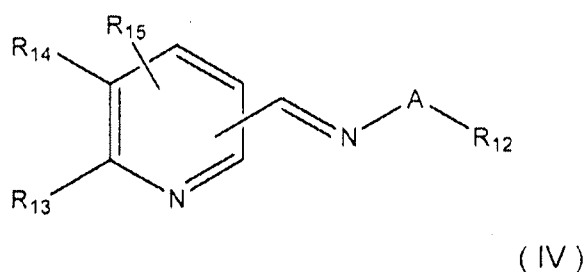
- v) grinding a compound of formula (IV) together with a reducing agent and a catalytic amount of acid, and
 - vi) monitoring the reduction reaction until the starting material has disappeared,
- thereby producing a compound of formula (VII):



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wherein R_{12} , R_{13} , R_{14} and R_{15} are as defined for formula (IV).

33. A method of preparing a compound of formula (IV)



wherein

A is a divalent linker selected from the group $-(CH_2)_{1-10}-$, $-aryl-$, $-(CH_2)_{1-5}-aryl-$, $-(CH_2)_{1-5}-aryl-(CH_2)_{1-5}-$, $-aryl-(CH_2)_{1-5}-$, $-aryl-NH-(CH_2)_{1-5}-$, $-aryl-NH-aryl-$, $-aryl-CONH-(CH_2)_{1-5}-$, $-aryl-CONH-aryl-$, $-aryl-CO-(CH_2)_{1-5}-$, and $-aryl-CO-aryl-$, where each aryl is optionally substituted;

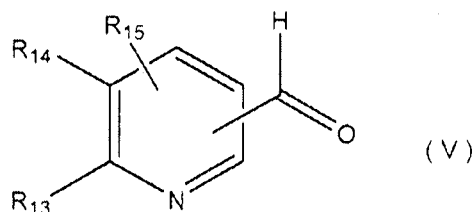
R_{12} is selected from $-CO_2H$, $-NH_2$ and OH ;

R_{13} and R_{14} are independently selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$; or R_{13} and R_{14} taken together with the carbon atoms to which they are attached form a 6

membered aromatic ring optionally substituted with one or more R_{15} ; and R_{15} is selected from hydrogen, $-C_{1-6}alkyl$, $-C_{2-6}alkenyl$, $-halo$, $-nitro$, $-OH$, $-OC_{1-6}alkyl$, $-SH$, $-Salkyl$, $-CN$, $-NH_2$, $-NH(C_{1-6}alkyl)$ and $-NH(C_{1-6}alkyl)_2$

said method comprising:

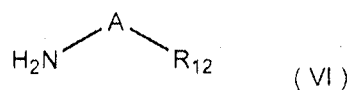
i) heating a solution of a carboxaldehyde of formula (V)



wherein R_{13} to R_{15} are as defined in formula (IV)

together with an amine compound of formula (VI)

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wherein A and R₁₂ are as defined in formula (IV), together with a catalytic amount
of an organic acid, under microwave conditions.

34. Use of a bacterial peptide selected from:

LEVVVNER [SEQ ID NO: 1]

YSYVDENGETK [SEQ ID NO: 2]

REEESAAAAEVEER [SEQ ID NO: 3] and

EMLIADGIDDNELLNSLA AVK [SEQ ID NO: 4]

as a bacterial marker that characterises *E. coli* or a strain of *E. coli*.

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FIGURE 1

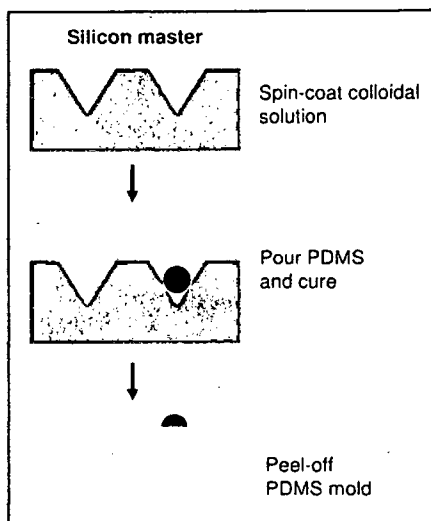


FIGURE 2



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FIGURE 3

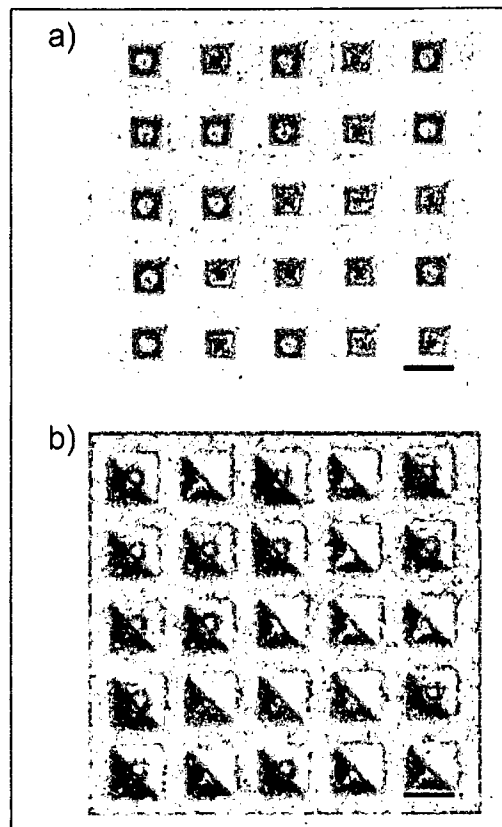
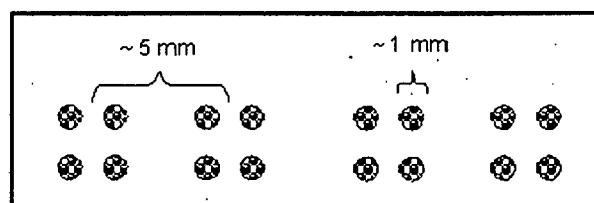


FIGURE 4



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FIGURE 5

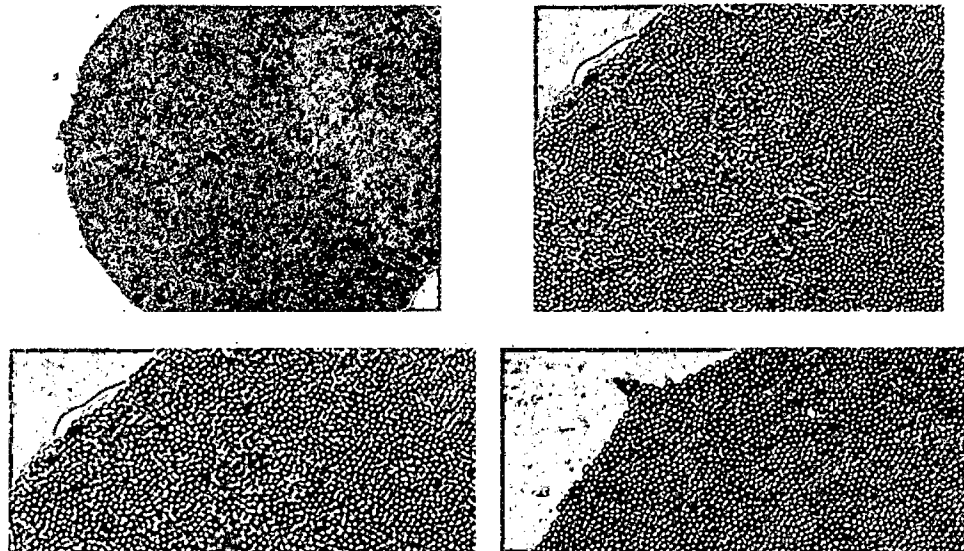
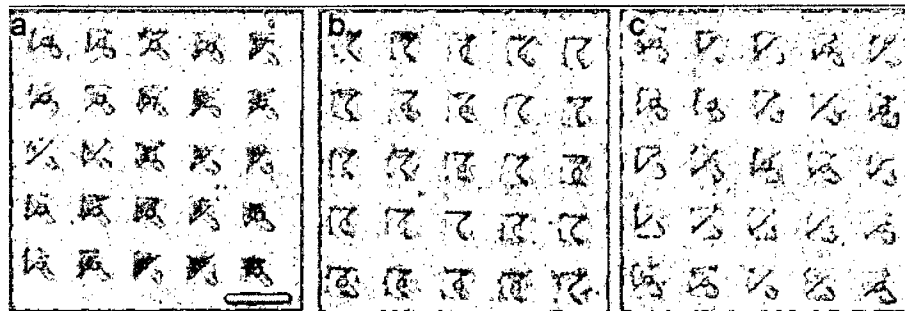


FIGURE 6



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FIGURE 7

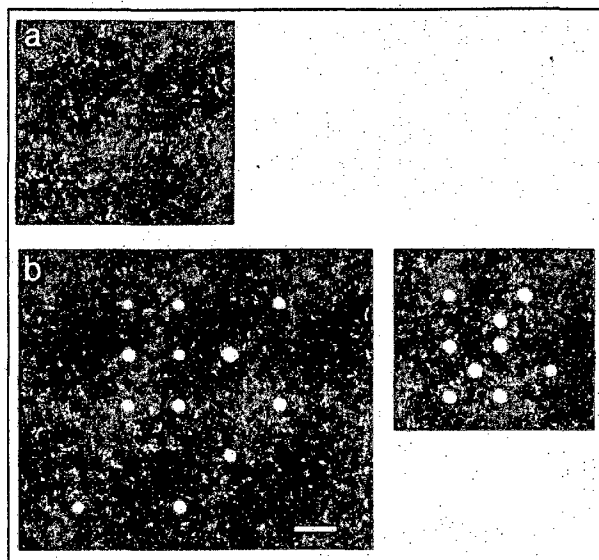
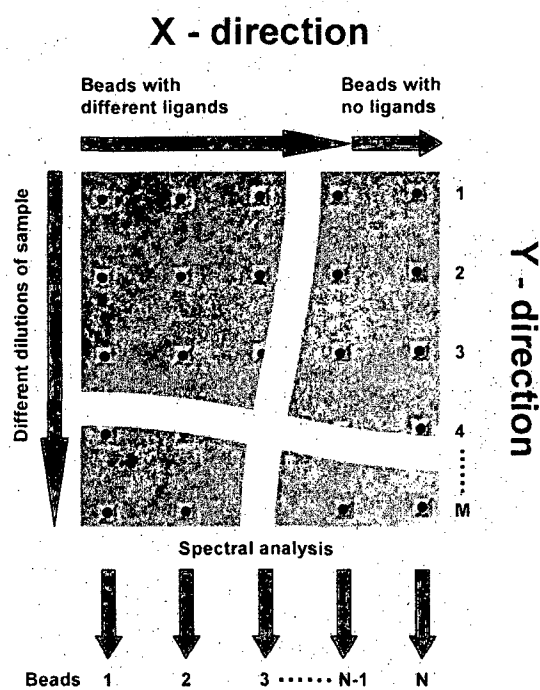
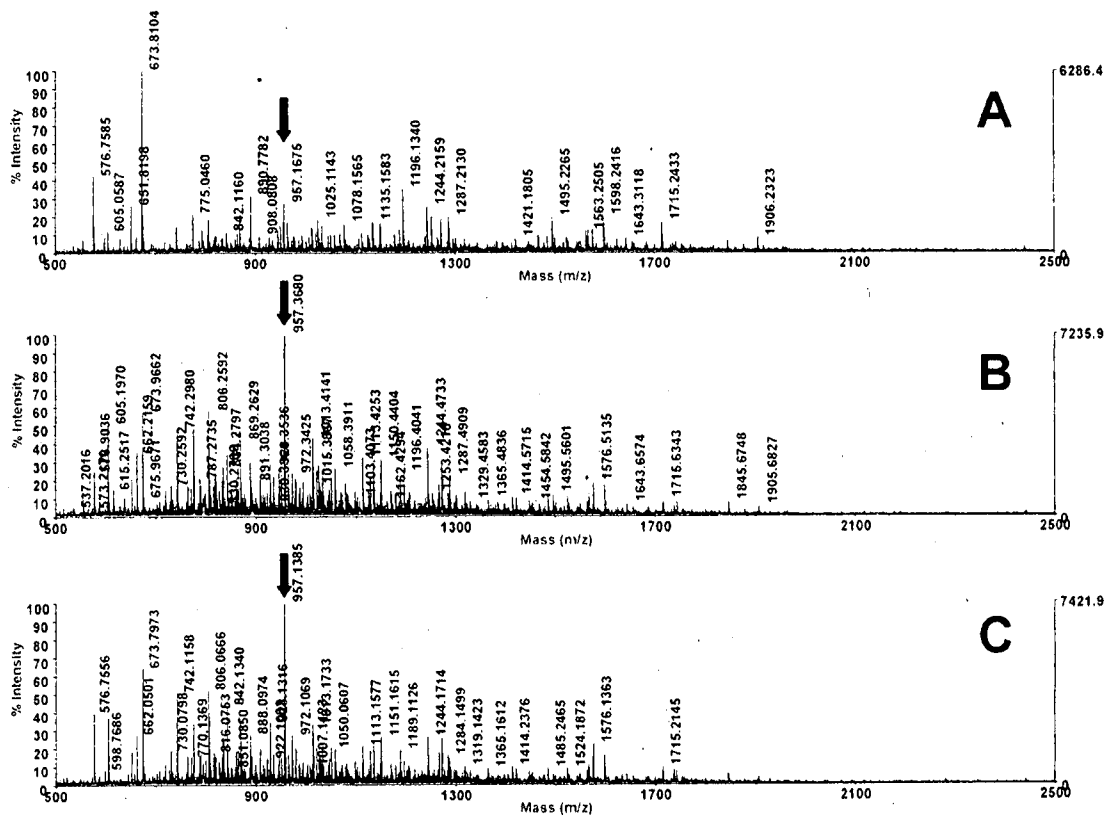


FIGURE 8



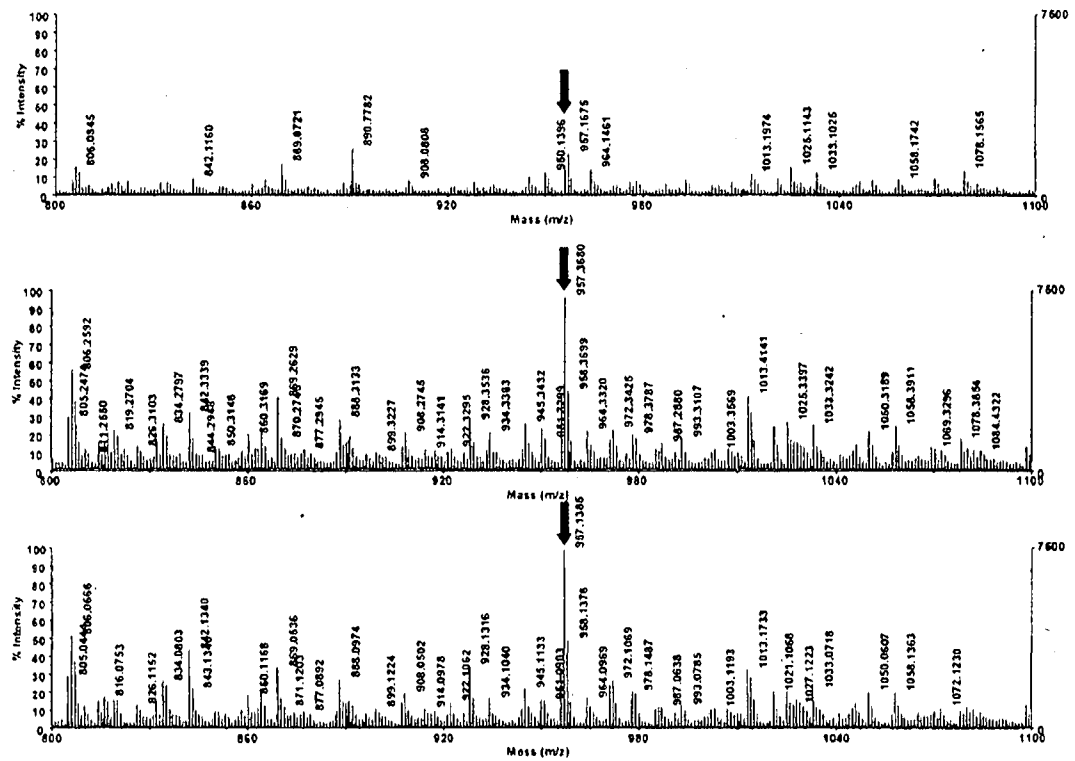
- 5/6 -

FIGURE 9



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FIGURE 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/000547

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C40B 40/04 (2006.01) *C07D 213/61* (2006.01) *C07D 215/18* (2006.01)
C07D 213/36 (2006.01) *C07D 215/06* (2006.01) *C07D 413/04* (2006.01)
C07D 213/53 (2006.01) *C07D 215/12* (2006.01) *C12Q 1/00* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Structure search based on claim 1 in Registry and CAplus. Additional keywords: library, array, bacteria, microbe, microorganism, bovine mastitis, microorganism, bacillus, staphylococcus, streptococcus

Keyword search in Medline, CAplus, Biosis, EPODOC, WPI using terms quinoline, pyridine, peptide, bovine mastitis, bacillus, staphylococcus, streptococcus, bacteria, E coli, array, library

BLAST search of SEQ ID NOS: 1-4 in ENSEMBL, Genbank, GenomeQuest patents, PDB, RefSeq, Swiss-Prot

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts file Registry: RN 24640-92-4, entered 16 November 1984 See compound 3-[(2-quinolinylmethylene)amino]-benzoic acid	29, 30



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 July 2011

Date of mailing of the international search report

28 July 2011

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/000547

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 260 592 A1 (MWG-BIOTECH AG), 27 November 2002 See SEQ ID NO: 4567	34
A	Naqvi A. <i>et al.</i> "Synthesis of Schiff Bases via Environmentally Benign and Energy-Efficient Greener Methodologies" E-Journal of Chemistry (2009) 6(S1): S75-S78 See page S76, Scheme 1	31-33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/000547

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- Claims 1-33 define compounds of formulas (I), (IA), (II), (IIA) and (III), the use of these compounds and libraries comprising these compounds, as well as methods of preparing compounds of formula (IV).
- Claim 34 defines the use of peptides of SEQ ID NOs: 1-4.

There is no structural relationship between the compounds in these groups of claims, therefore there is no "special technical feature" as required by PCT Rule 13 and the requirements for unity of invention are not satisfied *a priori*.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2011/000547

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
EP	1260592	US	2003119014
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. END OF ANNEX			