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(71) Applicant (for all designated States except US): **AUCKLAND UNISERVICES LIMITED** [NZ/NZ]; Level 10, 70 Symonds Street, Auckland, 1010 (NZ).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PATTERSON, Adam, Vorn** [GB/NZ]; 18 Manuka Road, Oneroa, Waiheke Island, 1081 (NZ). **SMALL, Jeffrey, Bruce** [NZ/NZ]; 17 Parkfield Terrace, Grafton, Auckland, 1023 (NZ). **DENNY, William, Alexander** [NZ/NZ]; 165 Gosamer Drive, Pakuranga, Auckland, 2010 (NZ). **WILSON, William, Robert** [NZ/NZ]; 199 Smith Road, RD2, Waiuku, 2682 (NZ). **SYDDALL, Sophie** [AU/NZ]; H4 - 19 Fleet Street, Eden Terrace, Auckland, 1021 (NZ).

ASHOORZADEH, Amir [NZ/NZ]; 37 Bentleigh Avenue, New Windsor, Auckland, 0600 (NZ).

(74) Agents: **ADAMS, Matthew, D.** et al.; A J Park, 6th Floor Huddart Parker Building, PO Box 949, Wellington, 6015 (NZ).

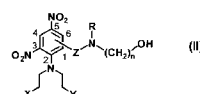
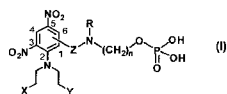
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(54) Title: NITROPHENYL MUSTARD ALCOHOLS, THEIR CORRESPONDING PHOSPHATES AND THEIR USE AS TARGETED CYTOTOXIC AGENTS



(57) Abstract: The invention provides nitrophenyl mustard compounds useful in cancer therapy as targeted cytotoxic agents, of Formula (I) and Formula (II): wherein X represents C1, Br, I, or OSO₂R₁, Y represents C1, Br, I, or OSO₂R₁, Z represents at any available ring position -CO- or -SO₂-, R represents: (1) a straight or branched chain C₁₋₆ alkyl group or -CH₂CH₂OMe, when X and Y are the same, or (2) a straight or branched chain C₃₋₆ alkyl group or -CH₂CH₂OMe when X and Y are different, R₁ represents a straight or branched chain C₁₋₆ alkyl group, and n is an integer from 1 to 6; and pharmaceutically acceptable salts thereof. Also provided are pharmaceutical compositions, comprising the compounds of Formula (I) and (II), and methods of treating cancer by administration of the compounds.

NITROPHENYL MUSTARD ALCOHOLS, THEIR CORRESPONDING PHOSPHATES AND THEIR USE AS TARGETED CYTOTOXIC AGENTS

FIELD OF THE INVENTION

5

The present invention relates to novel nitrophenyl mustard alcohols, to their corresponding phosphates, and to their use in cancer therapy as targeted cytotoxic agents.

BACKGROUND OF THE INVENTION

10

Symmetric (hetero)aromatic mustards are a class of compounds widely used in cancer therapy, both as direct cytotoxins (Feyns, *Analytical Profiles of Drug Substances*, 1984, 13 265), and as prodrugs for hypoxia (Palmer et al., *J. Med. Chem.*, 1990, 33, 112; Lee et al., *Bioorg. Med. Chem. Lett.*, 1998, 8, 1741), antibody-directed enzyme-prodrug therapy (ADEPT) (Springer et al., *J. Med. Chem.*, 1995, 38, 5051),
15 and gene-directed enzyme-prodrug therapy (GDEPT) (Niculescu-Duvaz et al., *J. Med. Chem.*, 2003, 46, 1690).

Asymmetric (hetero)aromatic mustards have also been described for use as prodrugs for hypoxia (Denny et al., *PCT Int. Appl.* WO 04033415 A1), in ADEPT (Pedley et al., *Cancer Res.*, 1999, 59,
20 3998), and GDEPT (Springer et al., *PCT Int. Appl.* WO01085960 A1).

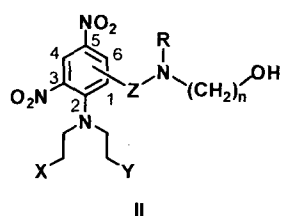
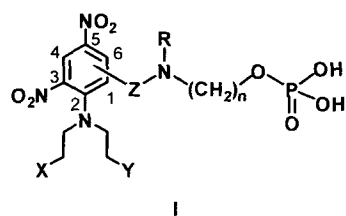
Dinitrobenzamide mustards bearing alcohol side chains pendant at a carboxamide (-CONH-) group and their phosphate derivatives have been described in WO 05042471. A process for preparing such mustards and intermediates produced by that process are described in WO 08030112 A1.

25

It is an object of the present invention to provide a specific class of nitrophenyl mustard alcohols and their corresponding phosphates, for use as targeted anticancer agents, or to at least to provide a useful alternative.

30 SUMMARY OF THE INVENTION

In a first aspect, the present invention provides compounds of Formula I and Formula II as shown below:



wherein

X represents Cl, Br, I, or OSO_2R_1 ,

5 Y represents Cl, Br, I, or OSO_2R_1 ,

Z represents at any available ring position $-\text{CO}-$ or $-\text{SO}_2-$,

R represents: (1) a straight or branched chain C_{1-6} alkyl group or $-\text{CH}_2\text{CH}_2\text{OMe}$, when X and Y are the same, or (2) a straight or branched chain C_{3-6} alkyl group or $-\text{CH}_2\text{CH}_2\text{OMe}$ when X and Y are different,

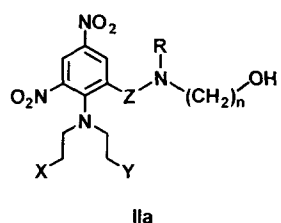
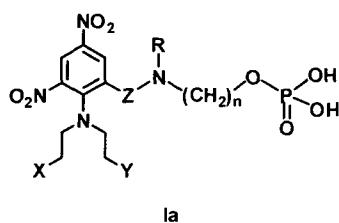
10 R_1 represents a straight or branched chain C_{1-6} alkyl group, and

n is an integer from 1 to 6;

and pharmaceutically acceptable salts thereof.

In preferred embodiments, the compounds are of Formula Ia or IIa as shown below:

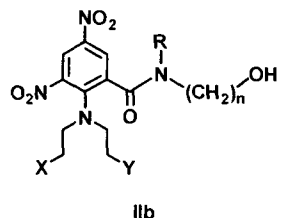
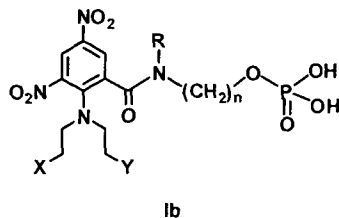
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wherein X, Y, Z, R and n are as defined for Formulae I and II.

20

In further preferred embodiments, the compounds are of Formula Ib or IIb as shown below:



wherein X, Y, R and n are as defined for Formulae I and II.

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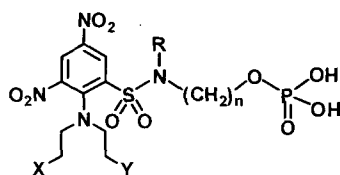
In certain embodiments, the compounds are of Formula Ib or IIb, where n is an integer from 1 to 3, preferably 2.

In certain embodiments, the compounds are of Formula Ib or IIb, where R is selected from methyl, ethyl, propyl and isopropyl when X and Y are the same, and from propyl, isopropyl and butyl when X and Y are different.

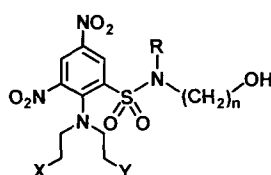
In certain embodiments, the compounds are of Formula Ib or IIb, where X and Y are both Br. In other embodiments, X and Y are both I. In other embodiments, X and Y are both OSO_2CH_3 .

In other embodiments, one of X and Y is Br or Cl and the other is OSO_2CH_3 .

In other embodiments, the compounds are of Formula Ic or IIc



Ic



IIc

wherein X, Y, R and n are as defined for Formulae I and II.

In certain embodiments, the compound is of Formula I and is selected from the following:

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (methyl)amino]ethyl dihydrogen phosphate (34),

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (ethyl)amino]ethyl dihydrogen phosphate (35),

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (isopropyl)amino]ethyl dihydrogen phosphate (36),

2-[{2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl} (methyl)amino]ethyl dihydrogen phosphate (37) and

2-[{2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl} (propyl)amino]ethyl dihydrogen phosphate (38); and pharmaceutically acceptable salts thereof.

In certain embodiments, the compound is of Formula II and is selected from the following:

- 2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (2),
- 5 2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (3),
- 2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (4),
- 10 2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (5),
- 2-[Bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (7),
- 2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (9),
- 15 2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (10),
- 2-((2-Bromoethyl)-2- {[(2-hydroxyethyl)(isopropyl)amino]carbonyl} -4,6-dinitroanilino)ethyl methanesulfonate (13),
- 20 2-((2-Bromoethyl)-2- {[(2-hydroxyethyl)(propyl)amino]carbonyl} -4,6-dinitroanilino)ethyl methanesulfonate (14),
- 2-((2-Chloroethyl)-2- {[(2-hydroxyethyl)(isopropyl)amino]carbonyl} -4,6-dinitroanilino)ethyl methanesulfonate (16),
- 25 2-((2-Chloroethyl)-2- {[(2-hydroxyethyl)(propyl)amino]carbonyl} -4,6-dinitroanilino)ethyl methanesulfonate (17),
- 30 2-(2- {[(2-Hydroxyethyl)(isopropyl)amino]carbonyl} {2-[(methylsulfonyl)oxy]ethyl} -4,6-dinitroanilino)ethyl methanesulfonate (19),
- 2-(2- {[(2-Hydroxyethyl)(propyl)amino]carbonyl} {2-[(methylsulfonyl)oxy]ethyl} -4,6-dinitroanilino)ethyl methanesulfonate (20),
- 35 2-[Bis(2-bromoethyl)amino]-*N*-(4-hydroxybutyl)-*N*-methyl-3,5-dinitrobenzamide (101),

2-[Bis(2-bromoethyl)amino]-*N*-(5-hydroxypentyl)-*N*-methyl-3,5-dinitrobenzamide (102),

2-[Bis(2-bromoethyl)amino]-*N*-(6-hydroxyhexyl)-*N*-methyl-3,5-dinitrobenzamide (103),

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2-[Bis(2-bromoethyl)amino]-*N*-(hydroxymethyl)-*N*-(3-hydroxypropyl)-3,5-dinitrobenzamide (104),

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(4-hydroxybutyl)-3,5-dinitrobenzamide (105),

10 2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(5-hydroxypentyl)-3,5-dinitrobenzamide (106), and

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(6-hydroxyhexyl)-3,5-dinitrobenzamide (107);

and pharmaceutically acceptable salts thereof.

15

In another aspect, the invention provides a pharmaceutical composition comprising a compound of Formula I or II as defined above or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

20 In a further aspect, the present invention provides the use of a compound of Formula I or II as defined above or a pharmaceutically acceptable salt thereof in the preparation of a medicament for the treatment of cancer.

In another aspect, the present invention provides a method for the production of an anti-cancer
25 effect in a warm-blooded animal such as a human, wherein the method comprises administering to the animal an effective amount of a compound of Formula I or II as defined above or a pharmaceutically acceptable salt thereof.

In a further aspect, the present invention provides a method for the production of an anti-cancer
30 effect in a cell, wherein the method comprises contacting the cell with an effective amount of a compound of formula I or II as defined above or a pharmaceutically acceptable salt thereof.

In another aspect, the present invention provides the use of a compound of Formula I or II as defined above or a pharmaceutically acceptable salt thereof in the preparation of a medicament for
35 the production of an anti-cancer effect in a warm-blooded animal such as a human.

In a further aspect, the present invention provides a method for the treatment of a cancer in a warm-blooded animal such as a human, which comprises administering to the animal an effective amount of a compound of Formula I or II as defined above or a pharmaceutically acceptable salt thereof.

The compound can be administered as a monotherapy, but will more usually be administered as part of a combination approach together with one or more other anti-cancer agents.

- 10 In certain embodiments, the method of treating cancer further includes the step of administering radiation treatment.

- 15 In a further aspect, the invention provides a method of ablating tumour cells, comprising the step of administering a compound of Formula I or Formula II as defined above or a pharmaceutically acceptable salt thereof to said cells in an amount effective to ablate those cells.

In certain embodiments of the methods described above, the compound of Formula I or II is administered as part of a combination treatment.

- 20 In one preferred approach, administration is in combination with a therapy that results in expression of an exogenous nitroreductase enzyme within, or therapeutically proximate to, a tumour.

- 25 In certain embodiments, the expression of the nitroreductase enzyme results from GDEPT (gene-directed enzyme prodrug therapy), such as VDEPT (virus-directed enzyme prodrug therapy), or CDEPT (*Clostridia*-directed enzyme prodrug therapy).

- 30 In certain embodiments, the expression of the nitroreductase enzyme results from GDEPT and the nitroreductase enzyme that is expressed is encoded by the *nfsB* gene of either *E. coli* or orthologous genes in *Clostridia* species.

In an alternative embodiment, an exogenous nitroreductase may be introduced by ADEPT (antibody-directed enzyme prodrug therapy).

DEFINITIONS

5 “Anti-cancer effects” include, but are not limited to, anti-tumour effects, the response rate, the time to disease progression and the survival rate. “Anti-tumour” effects include but are not limited to inhibition of tumour growth, tumour growth delay, regression of tumour, shrinkage of tumour, increased time to regrowth of tumour on cessation of treatment and slowing of disease progression.

10 “Effective amount” means an amount of a compound that, when administered to a subject for treating a cancer, is sufficient to effect such treatment for the cancer. The “effective amount” will vary depending on the cancer to be treated, the compound to be administered, the severity of the cancer treated, the age and relative health of the subject, the route and form of administration, whether the treatment is monotherapy or combination therapy, the judgement of the attending clinician, and other factors.

15 “Pharmaceutically acceptable”, means that which is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and neither biologically nor otherwise undesirable and includes that which is acceptable for veterinary as well as human pharmaceutical use.

20 “Pharmaceutically acceptable salts” of a compound means salts that are pharmaceutically acceptable, as defined herein, and that possess the desired pharmacological activity of the parent compound. Such salts include:

acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like; or formed with organic acids such as acetic
25 acid, methanesulfonic acid, maleic acid, tartaric acid, citric acid and the like; or

salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g. an alkali metal ion, an alkaline earth ion, or an aluminium ion; or coordinates with an organic or inorganic base. Acceptable organic bases include ethanolamine, diethanolamine, N-methylglucamine, triethanolamine and the like. Acceptable inorganic bases include aluminium
30 hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate and sodium hydroxide.

“Warm blooded animal” means any member of the mammalia class including, but not limited to humans, non-human primates such as chimpanzees and other apes and monkey species, farm animals such as cattle, horses, sheep, goats, and swine; domestic animals such as rabbits, dogs and
35 cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like.

BRIEF DESCRIPTION OF THE FIGURES

5 Figure 1 shows the correlation between lipophilicity (LogP) and cytotoxicity (IC_{50}) at low cell density, for certain compounds of the invention.

Figure 2 shows the correlation between lipophilicity (LogP) and cytotoxicity (C_{10}) against NTR-expressing 'activator' cells at high cell densities, for certain compounds of the invention.

10 Figure 3 shows the correlation between lipophilicity (LogP) and cytotoxicity (C_{10}) against NTR-naive 'target' cells when grown at high cell densities in the presence of 1% NTR-positive 'activator' cells, for certain compounds of the invention.

15 Figure 4 shows a human normal tissue microarray stained for aldo-keto reductase IC3 expression.

Figure 5 shows cell cytotoxicity data for a number of compounds of the invention, and indicates that incremental N-alkyl extension of C2 hydroxyalkyl carboxyamide sidechains diminishes AKR1C3 dependent cell sensitivity under aerobic conditions.

20

DETAILED DESCRIPTION OF THE INVENTION

Compounds of the invention

25 The present invention relates to a class of compounds for use as agents or drugs for cancer therapy and related methods. In particular, it provides a specific class of nitrophenyl mustards of Formula II and their corresponding phosphates of Formula I, as defined above, for use as targeted cytotoxic agents or bioreductive prodrugs.

30 The nitrophenyl mustards are relatively inactive in their nitro form. Without wishing to be bound by theory, it is believed that the nitrophenyl mustard compounds penetrate tumour tissue and are able to be selectively reduced to an active (cytotoxic) form by a range of nitroreductase enzymes. This active form is able to kill the tumour cells.

The nitroreductase enzyme may be an enzyme endogenous to the tumour cells, such as an enzyme expressed in hypoxic regions within the tumour, which reduces the nitrophenyl mustard compounds under hypoxic conditions. Alternatively, the nitroreductase may be an exogenous enzyme. Such an enzyme may be present within or therapeutically proximate to the tumour cells by expression of appropriately encoding nucleic acid that has been introduced into the patient. An approach for therapeutic introduction of enzyme-encoding nucleic acid is GDEPT (gene-directed enzyme prodrug therapy), which includes VDEPT (virus-directed enzyme prodrug therapy) and CDEPT (*Clostridia*-directed enzyme prodrug therapy). GDEPT is described, for example, in US 6,310,237 (Denny *et al*). VDEPT is described, for example, in *Proc. Natl. Acad. Sci. USA* (1991) 88, 8039 (Huber *et al*).

In an alternative embodiment, an exogenous nitroreductase can be introduced by ADEPT (antibody-directed enzyme prodrug therapy). This approach to cancer therapy is disclosed in WO 88/07378 and involves the administration of a monoclonal antibody-enzyme conjugate. The enzyme of the conjugate is one that is capable of reducing a prodrug and the monoclonal antibody is one that will bind to a tumour associated antigen. The prodrug compound is converted into an anti-tumour agent under the influence of the enzyme.

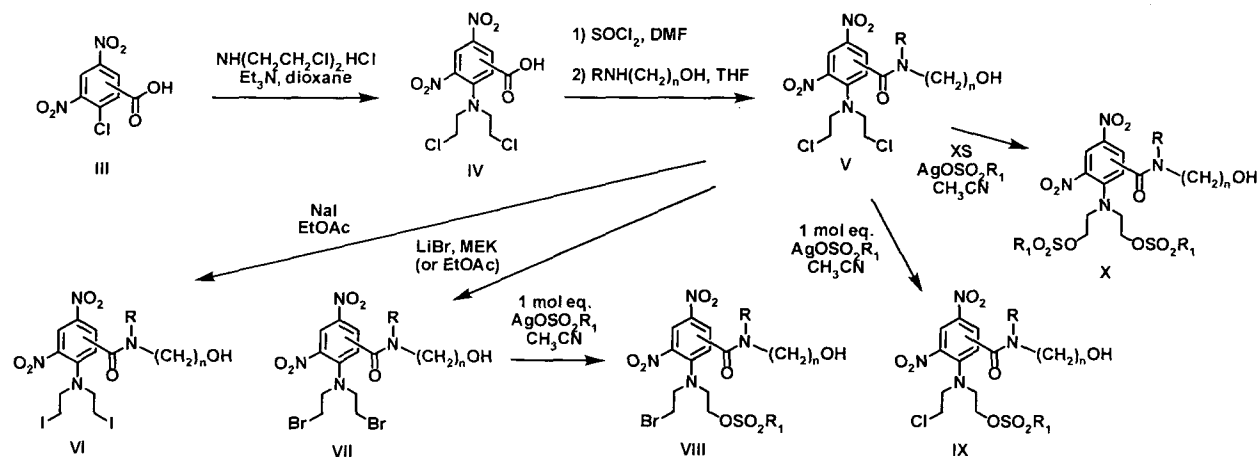
Preferably, however the mustard is reduced, the active form of the mustard (a cytotoxic metabolite) is capable of back diffusion (known as the bystander effect) to kill nitroreductase-naïve cells within the tumour. Mustards with good bystander effects are preferred.

Methods of preparing the compounds of the invention are described below.

Preparation of dinitrobenzamide mustard prodrugs of the invention

The preferred dinitrobenzamide mustard prodrug compounds of Formula I and II may be prepared employing methods analogous to those described in the literature (Atwell *et al*, PCT Int. Appl. WO 2008030112 A1; Yang *et al*, Tetrahedron, 2007, 63, 5470-5476; Atwell *et al*, J. Med. Chem. 2007, 50, 1197-1212; Denny *et al*, PCT Int. Appl. WO 2005042471 A1; Denny *et al*, PCT Int. Appl. WO 2004033415 A1; Friedlos *et al*, J. Med. Chem. 1997, 40, 1270-1275; Atwell *et al*, Anti-Cancer Drug Design, 1996, 11, 553-567; Palmer *et al*, J. Med. Chem. 1996, 39, 2518-2528; Palmer *et al*, J. Med. Chem. 1994, 37, 2175-84.)

In general terms, the preferred dinitrobenzamide mustard prodrug compounds of Formula II may be prepared as shown in scheme 1 from the known 3-chloro-2,6-dinitrobenzoic acid (Palmer et al, J. Med. Chem. 1996, 39, 2518-2528), or the commercially available 2-chloro-3,5-dinitrobenzoic acid and 5-chloro-2,4-dinitrobenzoic acid, respectively. Reaction with *N,N*-bis(2-chloroethyl)amine hydrochloride in dioxane employing triethylamine as base provides the dichloro mustard acids (IV). Conversion of these to their respective acid chlorides and then reaction with secondary hydroxyalkyl amines, which are themselves commercially available or readily prepared using the reductive amination method of Saavedra (J. Org. Chem. 1985, 50, 2271), provides the dinitrobenzamides (V). Sodium iodide or lithium bromide mediated halogen exchange then provides the symmetrical diiodo and dibromo mustards VI and VII, respectively. The asymmetric halogen/alkyl sulfonate mustards (VIII, IX) can be prepared from their symmetrical counterparts by reaction with approximately one equivalent of a silver alkylsulfonate salt in an appropriate solvent, such as acetonitrile. The bis-alkyl sulfonate mustards (X) can be prepared from their symmetrical counterparts (for example V) by reaction with an excess of a silver alkylsulfonate salt in an appropriate solvent, such as acetonitrile.

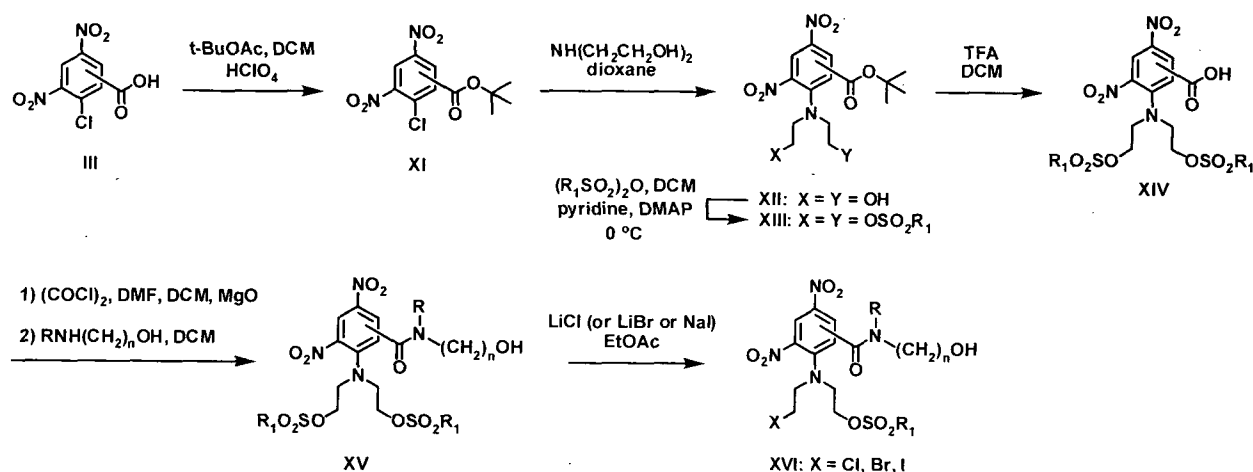


Scheme 1

As an alternative method, in general terms, the preferred dinitrobenzamide mustard prodrug compounds of Formula II may be prepared as shown in scheme 2 from the known 3-chloro-2,6-dinitrobenzoic acid (Palmer et al, J. Med. Chem. 1996, 39, 2518-2528), or the commercially available 2-chloro-3,5-dinitrobenzoic acid and 5-chloro-2,4-dinitrobenzoic acid, respectively. Reaction with tert-butyl acetate in the presence of perchloric acid provides the respective tert-butyl esters (XI).

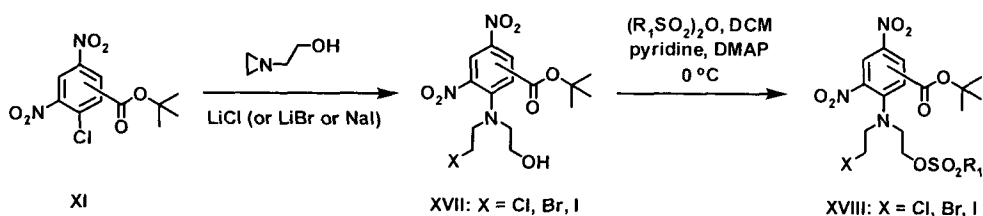
Reaction of these with diethanolamine in dioxane then affords the respective diols (XII). Conversion of these to the bis-alkylsulfonates (XIII) can be achieved utilizing the appropriate alkylsulfonic anhydride in the presence of pyridine and catalytic dimethylaminopyridine (DMAP).

Trifluoroacetic acid mediated tert-butyl ester hydrolysis, followed by conversion of the intermediate acids (XIV) to their respective acid chlorides, employing oxalyl chloride in the presence of magnesium oxide, and then subsequent reaction of these acid chlorides with secondary hydroxyalkyl amines, that are themselves commercially available or readily prepared using the reductive amination method of Saavedra (J. Org. Chem. 1985, 50, 2271), provides the bis-alkylsulfonate dinitrobenzamides (XV). Lithium chloride, lithium bromide or sodium iodide mediated partial alkyl sulfonate displacement, followed by the appropriate chromatography, then provides the asymmetric halogen/alkyl sulfonate mustards (XVI).



Scheme 2

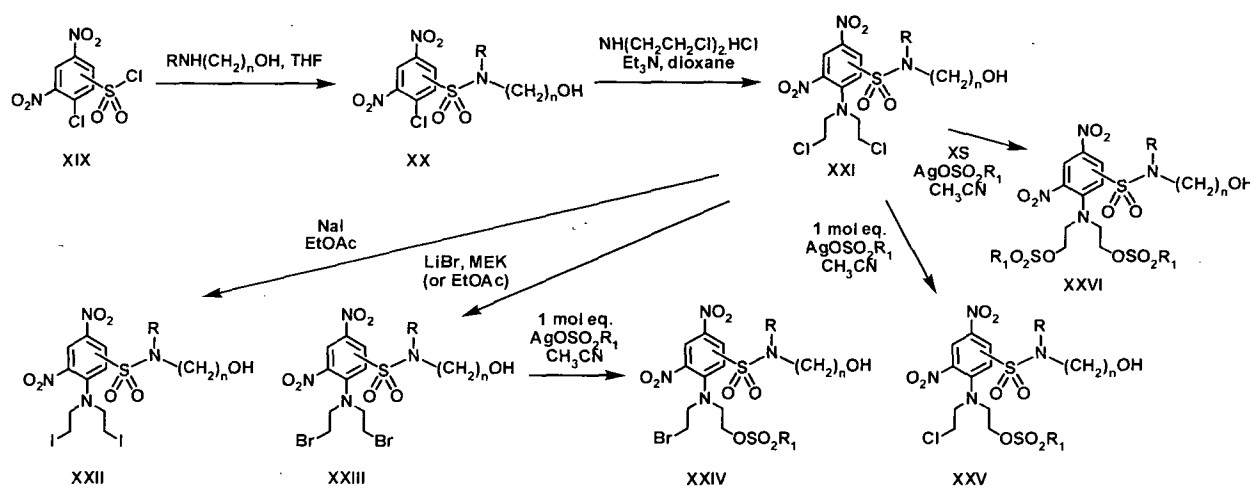
As an alternative method, in general terms, the preferred asymmetric dinitrobenzamide mustard prodrug compounds of Formula II may also be prepared following the methodology of Yang et al (Tetrahedron, 2007, 63, 5470-5476) as shown in scheme 3. The above described tert-butyl esters (XI) can be reacted with aziridine ethanol in the presence of a metal halide (LiCl , LiBr or NaI) to provide the haloethyl half-mustards (XVII), which can be derivatised to the halo/alkylsulfonate mustards (XVIII) by reaction with the appropriate alkylsulfonic anhydride in the presence of pyridine and catalytic dimethylaminopyridine (DMAP). Tert-butyl ester deprotection, acid chloride formation and amide coupling with secondary hydroxyalkyl amines, as described above, then provides the asymmetric halogen/alkyl sulfonate mustards of Formula II.



Scheme 3

Preparation of dinitrobenzenesulfonamide mustard prodrugs of the invention

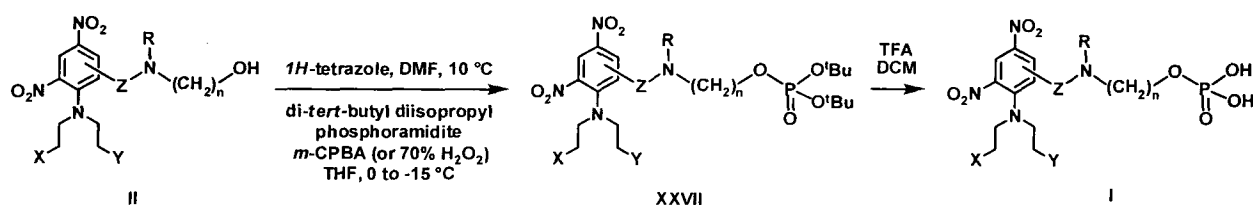
In general terms, the preferred dinitrobenzenesulfonamide mustard prodrug compounds of Formula II may be prepared as shown in scheme 4 from the known 2-chloro-3,5-dinitrobenzenesulfonyl chloride and 5-chloro-2,4-dinitrobenzenesulfonyl chloride (McNally et al, 1944, US 2358465; Herbert and Holliman, Tetrahedron, 1965, 21, 663-75), respectively. Reaction with secondary hydroxyalkyl amines, that are themselves commercially available or readily prepared using the reductive amination method of Saavedra (J. Org. Chem. 1985, 50, 2271), provides the dinitrobenzenesulfonamides (XX). Chlorine displacement with *N,N*-bis(2-chloroethyl)amine hydrochloride in dioxane employing triethylamine as base, provides the dichloro mustards (XXI). Sodium iodide or lithium bromide mediated halogen exchange then provides the symmetrical diiodo and dibromo mustards XXII and XXIII, respectively. The asymmetric halogen/alkyl sulfonate mustards (XXIV, XXV) can be prepared from their symmetrical counterparts by reaction with approximately one equivalent of a silver alkylsulfonate salt in an appropriate solvent, such as acetonitrile. The bis-alkyl sulfonate mustards (XXVI) can be prepared from their symmetrical counterparts by reaction with an excess of a silver alkylsulfonate salt in an appropriate solvent, such as acetonitrile.



Scheme 4

Preparation of phosphates of dinitrobenzamide and dinitrobenzenesulfonamide mustard prodrugs of the invention

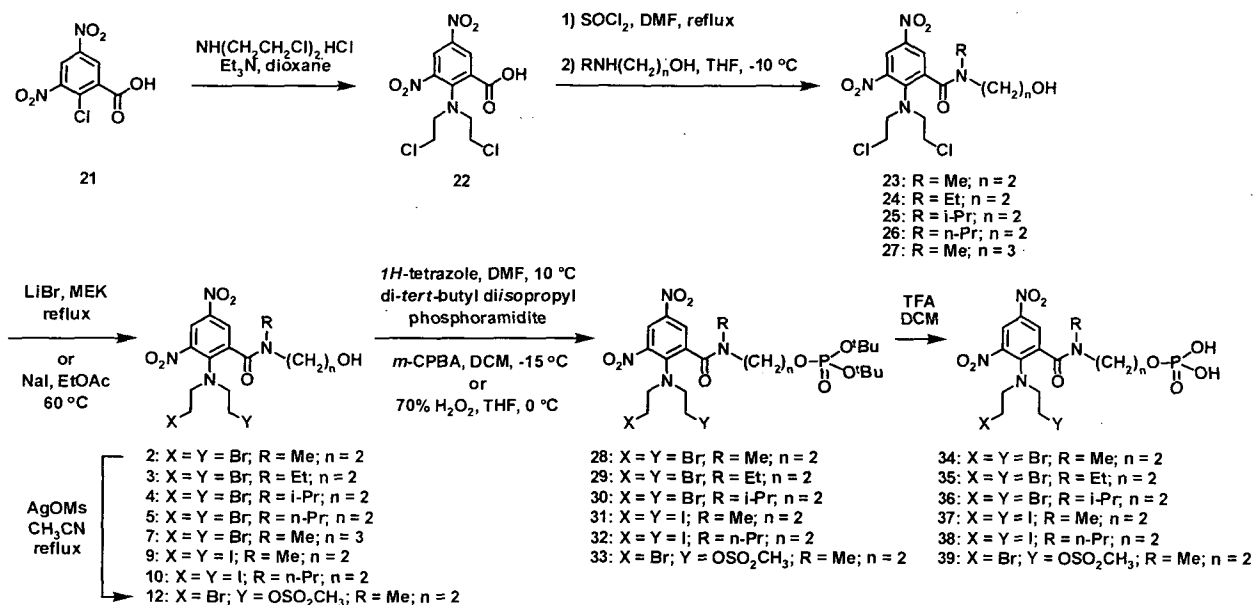
- 5 In general terms, phosphates of Formula I may be prepared as shown in scheme 5 from the preferred prodrug compounds of Formula II by reaction of these alcohol derivatives with di-*tert*-butyl diisopropylphosphoramidite utilizing 1*H*-tetrazole as the base, followed by oxidation with either *m*-chloroperoxybenzoic acid (*m*-CPBA) or 70% aqueous hydrogen peroxide, to provide the di-*tert*-butylphosphate ester intermediates (XXVII). Acid mediated hydrolysis, employing trifluoroacetic acid (TFA) in dichloromethane, then provides the phosphates of Formula I as their free acids.



Scheme 5

- 15 Scheme 6 below illustrates the preparation of a number of dinitrobenzamide mustard prodrug compounds of Formula I and II according to the invention. Reaction of the commercially available 2-chloro-3,5-dinitrobenzoic acid (21) with *N,N*-bis(2-chloroethyl)amine hydrochloride in dioxane employing triethylamine as base provides the dichloro mustard acid (22). Conversion of this to the acid chloride and then reaction with secondary hydroxyalkyl amines, which are themselves commercially available or readily prepared using the reductive amination method of Saavedra (J. Org. Chem. 1985, 50, 2271), provides the dinitrobenzamides (23-27). Lithium bromide or sodium iodide mediated halogen exchange then provides the symmetrical dibromo and diiodo mustards (2-5, 7 and 9, 10, respectively). The asymmetric bromo/methyl sulfonate mustard (12) was prepared as a reference compound, from its symmetrical dibromo counterpart (2) by reaction with one equivalent of silver mesylate in acetonitrile. Phosphates of Formula I were prepared from the alcohols (2-4, 9, 10) by reaction with di-*tert*-butyl diisopropylphosphoramidite utilizing 1*H*-tetrazole as the base, followed by oxidation with either *m*-chloroperoxybenzoic acid (*m*-CPBA) or 70% aqueous hydrogen peroxide, to provide the di-*tert*-butylphosphate ester intermediates (28-32). Acid mediated hydrolysis, employing trifluoroacetic acid (TFA) in dichloromethane, then provides the phosphates (34-38) of Formula I as their free acids. The phosphate (39) of reference compound (12) was prepared similarly, as described above.

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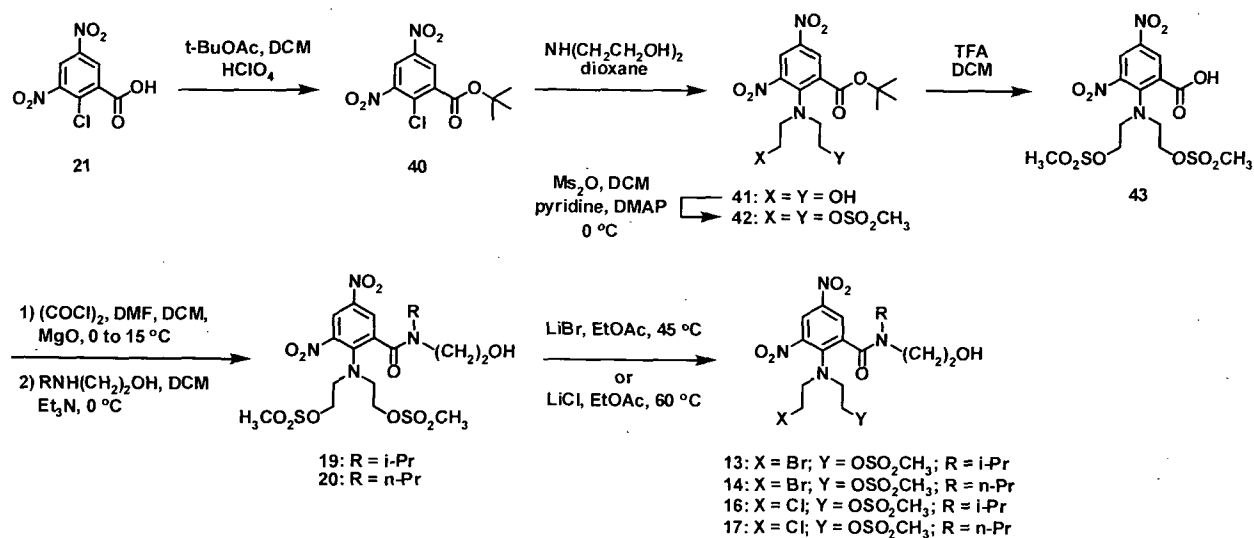


Scheme 6

- 5 Preparation of comparative compounds (1, 6, 8 and 11), unsubstituted at the carboxamide nitrogen, was as described by Denny et al (PCT Int. Appl. WO 2005042471 A1; PCT Int. Appl. WO 2004033415 A1).

Schemes 7 below illustrates an alternative preparation of a number of dinitrobenzamide mustard prodrug compounds of formula II according to the invention. Conversion of the commercially available 2-chloro-3,5-dinitrobenzoic acid (21), via the known compounds 40 and 41, to the bis-methylsulfonate (42) was achieved following the method of Atwell et al (Journal of Labelled Compounds and Radiopharmaceuticals, 2007, 50, 7-12). Trifluoroacetic acid mediated tert-butyl ester hydrolysis, followed by conversion of the intermediate acid (43) to its acid chloride, employing oxalyl chloride in the presence of magnesium oxide, and then subsequent reaction of this acid chloride with 2-(isopropylamino)ethanol and 2-(propylamino)ethanol respectively, gave bis-mesylates 19 and 20. Lithium bromide and lithium chloride, mediated partial alkyl sulfonate displacement, followed by chromatography, then gave the asymmetric bromo/methyl sulfonates (13, 14) and chloro/methylsulfonates (16, 17), respectively.

15



Scheme 7

5 Therapeutic uses of the compounds of the invention

The compounds of the Formulae I and II of the present invention can be used in the treatment of cancer of the human or animal body. In particular embodiments, the treatment may be of any cancer type that includes hypoxic regions, as the mustards of Formula II are reduced by enzymes present in such regions. For example, the cancers treated may be solid tumours, such as ovarian, colon, brain, thyroid, pancreas, bladder, breast, prostate, lung (such as small cell lung tumour cells and large cell lung carcinoma), cervical and skin cancer. Alternatively, the cancer may be leukaemia, multiple myeloma or lymphoma. All of these cancers present with hypoxic regions, particularly where tumours are growing or have become large.

The compounds of the invention can be administered in the form of pharmaceutical compositions, containing one or more compounds of the invention in combination with one or more pharmaceutically acceptable carriers.

The pharmaceutically acceptable carrier(s) should be non-toxic and not interfere with the efficacy of the active ingredient. The precise nature of the carrier will depend on the route of administration, which can be oral, or parenteral, including intravenous, cutaneous, subcutaneous, intramuscular, intravascular or by infusion.

Pharmaceutical compositions suitable for oral administration can be in tablet, capsule, powder or liquid form. A tablet may comprise one or more solid carriers and/or adjuvants. A capsule may include a

solid carrier such as gelatin. Liquid pharmaceutical compositions may comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solutions or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

5

For parenteral injection, the pharmaceutical composition may conveniently be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has a suitable pH, isotonicity and stability. Those of skill in the art are able to prepare suitable solutions using, for example, isotonic vehicles such as sodium chloride injection, Ringer's injection, and Lactated Ringer's injection.

10 Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included as required.

The exact dose of the compound to be administered will be at the discretion of the physician, taking into account the type of cancer, the therapeutic approach (monotherapy or combination therapy) and the overall condition and needs of the patient. Typical doses and administration schedules will be
15 determined by experience in clinical trials. Total doses are expected to be in the range from about 0.1 to 200 mg/kg per subject, such as about 10 mg/kg per subject. The amount of compound administered may be between about 20% and 100% of the maximum tolerated dose of the subject.

The compounds of Formula I and II can be used as single agents or in combination with one or more
20 other cytotoxic or other therapeutic agents or therapies, especially those that are relatively ineffective against hypoxic cells, such as radiation therapy. Where such other agents and/or radiotherapy are administered in combination with a compound of the invention, the radiation and/or other agents may be administered before, during or after administration of the compound of Formula I and II.

25 In certain embodiments, the compound of Formula I or II may be administered for activation (reduction) by an exogenous nitroreductase enzyme. The exogenous nitroreductase enzyme will be present within or therapeutically proximate the tumour preferably as the result of introduction and expression of nucleic acid encoding it. Introduction of the nucleic acid may be part of GDEPT (gene-directed enzyme prodrug therapy), which includes VDEPT (virus-directed enzyme prodrug
30 therapy) and CDEPT (*Clostridia*-directed enzyme prodrug therapy). The nitroreductase enzyme expressed as part of this approach can, for example, be encoded by the *nfsB* gene of either *E. coli* or by orthologous genes in *Clostridia* species. However, it will be appreciated that any exogenous nitroreductase having the ability to reduce the mustard prodrugs of the invention can be encoded and introduced. Examples of such nitroreductases include: (1) *E.coli* NfsA: Vass SO, Jarrom D,
35 Wilson WR, Hyde EI, Searle PF. *E. coli* NfsA: an alternative nitroreductase for prodrug activation

gene therapy in combination with CB1954. *Br J Cancer*, 2009;100: 1903-11; (2) *E.coli* YieF: Barak Y, Thorne SH, Ackerley DF, Lynch SV, Contag CH, Matin A. New enzyme for reductive cancer chemotherapy, YieF, and its improvement by directed evolution. *Mol Cancer Ther* 2006;5: 97-103; and (3) *B.amyloliquefaciens* YwrO: Anlezark GM, Vaughan T, Fashola-Stone E, Michael NP, Murdoch H, Sims MA, Stubbs S, Wigley S, Minton NP. *Bacillus amyloliquefaciens* orthologue of *Bacillus subtilis* ywrO encodes a nitroreductase enzyme which activates the prodrug CB 1954. *Microbiology* 2002; 14: 297-306.

In an alternative embodiment, an exogenous nitroreductase can be introduced by ADEPT (antibody-directed enzyme prodrug therapy).

A GDEPT approach is preferred. It is envisaged that the use of GDEPT in combination of a compound of the present invention may be particularly beneficial when the cancer to be treated is one in which tumour hypoxia is a less reliable target, meaning that activation by endogenous enzymes is insufficient on its own.

Without wishing to be bound by theory, it is believed that compounds of Formula I, upon administration to a subject, are hydrolysed in vivo to form compounds of Formula II, and that compounds of Formula II are reduced selectively under hypoxic conditions by endogenous nitroreductases (or by introduced nitroreductases, for example, when GDEPT is used in conjunction with administration of the compounds) to form cytotoxic amino compounds.

The invention will now be described in more detail with reference to the following non-limiting experimental section.

EXPERIMENTAL

1. SYNTHESIS

1.1 Chemical synthesis

Combustion analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined using either an Electrothermal Model 9200 and are as read. ¹H NMR spectra were measured either on a Bruker Avance-400 spectrometer and are referenced to Me₄Si. High resolution mass spectra were recorded on a Varian VG-70SE spectrometer at nominal 5000 resolution. Mass spectrometry was performed on a ThermoFinnigan

MSQ single quadrupole mass spectrometer. Mass detection was performed with an APCI source, using simultaneous positive and negative ion acquisition. Unless otherwise indicated, compounds were purified by flash column chromatography on Silica gel 60 support (Scharlau, 230-400 mesh ASTM), using the indicated eluants.

5

1.1.1 Preparation of the N-alkyl carboxamide mustard alcohols

1.1.1.1 2-[Bis(2-bromoethyl)amino]-N-(2-hydroxyethyl)-N-methyl-3,5-dinitrobenzamide (2)

10 A mixture of 2-chloro-3,5-dinitrobenzoic acid (**21**) (25.0 g, 10.10 mol), bis(2-chloroethyl)amine hydrochloride (36.2 g, 0.20 mol) and Et₃N (42.3 mL, 0.30 mol) in dioxane (400 mL) was stirred at 25 °C for 72 h, then concentrated below 30 °C to 150 mL and diluted with excess 1N aqueous HCl. Prolonged cooling at 0 °C provided a solid which was collected by filtration and chromatographed on silica gel, eluting with EtOAc/petroleum ether (3:1). The product thus obtained was triturated
15 with iPr₂O to give 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**22**) (26.9 g, 75%) as a yellow solid: mp (MeOH/H₂O) 142-143 °C; ¹H NMR [(CD₃)₂SO] δ 14-16 (br s, 1 H), 8.81 (d, *J* = 2.8 Hz, 1 H), 8.59 (d, *J* = 2.8 Hz, 1 H), 3.70 (t, *J* = 6.7 Hz, 4 H), 3.46 (t, *J* = 6.7 Hz, 4 H).

A stirred solution of 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**22**) (15.0 g, 42.6 mmol) in
20 SOCl₂ (100 mL) and DMF (5 drops) was heated under reflux for 3 h, then cooled to room temperature and the excess SOCl₂ was removed under reduced pressure. The resulting crude 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoyl chloride was dissolved in THF (50 mL) and added slowly to a stirred solution of 2-(methylamino)ethanol (9.60 g, 12.8 mmol) in THF (80 mL) at -10 °C. The mixture was stirred at -10 °C for 5 min, then acidified with 0.5 N aqueous HCl (110 mL),
25 concentrated to half volume under reduced pressure and extracted with EtOAc (2x). The combined organic phases were washed with brine, dried and evaporated, and the residue was chromatographed on silica gel, eluting with EtOAc, followed by crystallisation from EtOAc/iPr₂O, to give 2-[bis(2-chloroethyl)amino]-N-(2-hydroxyethyl)-N-methyl-3,5-dinitrobenzamide (**23**) (15.82 g, 91%): mp 136-137 °C; ¹H NMR [(CD₃)₂SO] δ (mixture of rotamers) 8.71, 8.69 (2d, *J* = 2.8 Hz, 1 H), 8.39, 8.34
30 (d, *J* = 2.8 Hz, 1 H), 4.90-4.81 (m, 1 H), 3.73-3.29, 3.18-3.11 (2m, 12 H), 3.03, 2.94 (2s, 3 H). Anal. Calcd for C₁₄H₁₈Cl₂N₄O₆: C, 41.1; H, 4.4; N, 13.7; Cl, 17.3%; found: C, 41.4; H, 4.6; N, 13.8; Cl, 17.3%.

A mixture of **23** (20.0 g, 49 mmol) and LiBr (63.7 g, 730 mmol) in dry 3-methyl-2-butanone (methyl
35 ethyl ketone; MEK) (150 mL) was stirred at reflux for 3 h, then concentrated under reduced

pressure to remove most of the solvent. The residue was partitioned between EtOAc and water and the organic layer was washed with 10% aqueous NaI, dried and evaporated under reduced pressure. The residue was treated with further LiBr (63.7 g, 730 mmol) as above and worked up to provide crude product. A solution of this in EtOAc was filtered through a plug of silica gel, followed by
5 crystallisation from EtOAc, to give 2-[bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (**2**) (17.44 g, 72%) as a yellow solid: mp 130-132 °C; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.69 (2d, *J* = 2.8 Hz, 1 H), 8.40, 8.34 (2d, *J* = 2.8 Hz, 1 H), 4.85 (br s, 1 H), 3.70-3.33, 3.18-3.11 (2m, 12 H), 3.03, 2.95 (2s, 3 H). Anal. Calcd for C₁₄H₁₈Br₂N₄O₆: C, 33.8; H, 3.6; N, 11.3%; found: C, 34.2; H, 3.9; N, 11.4%.

10 1.1.1.2 2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (**3**)

A stirred solution of 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**22**) (1.50 g, 4.26 mmol) in SOCl₂ (15 mL) and DMF (3 drops) was heated under reflux for 3 h, then cooled to room
15 temperature and the excess SOCl₂ was removed under reduced pressure. The resulting crude 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoyl chloride was dissolved in THF (12 mL) and slowly added to a stirred solution of 2-(ethylamino)ethanol (prepared according to the literature procedure; J.Org.Chem. 1985, 50, 2271) (1.14 g, 12.79 mmol) in THF (12 mL) at -10 °C. The mixture was stirred for 20 min at -10 °C, then treated with aqueous HCl (0.5 M, 12 mL) to pH ~ 3 before being
20 extracted with EtOAc. The combined organic layers were washed with brine, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) to give 2-[bis(2-chloroethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (**24**) (1.21 g, 67%) as a yellow solid: m.p. 92-93 °C; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70, 8.69 (2d, *J* = 2.2 Hz, 1H), 8.38, 8.29 (2d, *J* = 2.2 Hz, 1H),
25 4.84 (s, br, 1H), 3.78-3.68 (m, 7H), 3.58-3.42 (m, 4H), 3.37-3.21 (m, 3H), 1.19, 1.06 (2t, *J* = 6.9 Hz, 3H). Anal. Calcd for C₁₅H₂₀Cl₂N₄O₆: C, 42.6; H, 4.8; N, 13.2; found: C, 42.7; H, 4.7; N, 13.4.

A mixture of 2-[bis(2-chloroethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (**24**) (1.13 g, 2.67 mmol) and LiBr (4.64 g, 53.46 mmol) in MEK (10 mL) was heated under reflux with
30 stirring for 3 h, then concentrated under reduced pressure to dryness. The residue was dissolved in EtOAc, washed with deionized water, 10% aqueous NaBr, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was resubmitted to LiBr (4.64 g, 53.46 mmol) in MEK (10 mL) followed by the above workup to provide the crude product, which was then purified by flash
column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1) to give 2-[bis(2-
35 bromoethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (**3**) (1.17 g, 86%) as a yellow

solid: m.p. 105-107 °C; ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.69 (2d, *J* = 2.8 Hz, 1H), 8.40, 8.30 (2d, 2.8 Hz, 1H), 4.86-4.814 (m, 1H), 3.80-3.62 (m, 3H), 3.60-3.52 (m, 7H), 3.46-3.17 (m, 4H), 1.19, 1.07 (2t, *J* = 7.07 Hz, 3H). Anal. Calcd for C₁₅H₂₀Br₂N₄O₆·0.1MEK: C, 35.8; H, 4.1; N, 10.7%; found: C, 35.7; H, 3.9; N, 10.7%.

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1.1.1.3 2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (4)

A stirred solution of 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (22) (1.50 g, 4.26 mmol) in SOCl₂ (15 mL) and DMF (3 drops) was heated under reflux for 3 h, then cooled to room
10 temperature and the excess SOCl₂ was removed under reduced pressure. The resulting crude 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoyl chloride was dissolved in THF (12 mL) and slowly added to a stirred solution of 2-(isopropylamino)ethanol (1.38 g, 13.39 mmol) in THF (12 mL) at -10 °C. The mixture was stirred for 20 min at -10 °C, then treated with aqueous HCl (0.5 M, 12 mL) to pH ~ 3 before being extracted with EtOAc. The combined organic layers were washed with
15 brine, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) to give 2-[bis(2-chloroethyl) amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (25) (1.01 g, 54%) as a yellow solid: m.p. 110-111 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.36, 8.25 (2d, *J* = 2.8 Hz, 1H), 4.80, 4.74 (2t, *J* = 5.7 Hz, 1H), 4.22, 3.82 (2m, 1H), 3.76-
20 3.65 (m, 4H), 3.58-3.35 (m, 5H), 3.33-3.18 (m, 3H), 1.35 (d, *J* = 6.7 Hz, 3H), 1.16 (2d, *J* = 8.4, 6.5 Hz, 3H). Anal. Calcd for C₁₆H₂₂Cl₂N₄O₆: C, 44.0; H, 5.1; N, 12.8%; found: C, 44.2; H, 5.2; N, 12.5%.

A mixture of 2-[bis(2-chloroethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (25) (1.0 g, 2.29 mmol) and LiBr (3.97 g, 45.74 mmol) in MEK (20 mL) was heated under reflux
25 with stirring for 3 h, then concentrated under reduced pressure to dryness. The residue was dissolved in EtOAc, washed with deionized water and then 10% aqueous NaBr before being dried with Na₂SO₄ and concentrated under reduced pressure. The residue was resubmitted to LiBr (3.97 g, 45.74 mmol) in MEK (20 mL) and then the above workup to provide the crude product, which was then purified by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1) to give
30 2-[bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (4) (960 mg, 80%) as a yellow solid: m.p. 110-112 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.72, 8.69 (2d, *J* = 2.8 Hz, 1H), 8.38, 8.26 (2d, 2.8 Hz, 1H), 4.81, 4.75 (2t, *J* = 5.8 Hz, 1H), 4.22, 4.18 (2m, 1H), 3.78-3.72 (m, 1H), 3.61-3.49 (m, 6H), 3.48-3.24 (m, 4H), 3.21 (t, *J* = 5.8 Hz, 1H), 1.35 (d, *J* = 6.8 Hz, 3H), 1.17 (2d, *J* = 6.5, 2.7 Hz, 3H). Anal. Calcd for C₁₆H₂₂Br₂N₄O₆: C, 36.5; H, 4.2; N, 10.7%; found:
35 C, 36.8; H, 4.2; N, 10.6%.

1.1.1.4 2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (5)

A stirred solution of 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**22**) (5.00 g, 14.20 mmol) in
5 SOCl₂ (50 mL) and DMF (5 drops) was heated under reflux for 3 h, then cooled to room
temperature and the excess SOCl₂ was removed under reduced pressure. The resulting crude 2-
[bis(2-chloroethyl)amino]-3,5-dinitrobenzoyl chloride was dissolved in THF (40 mL) and slowly
added to a stirred solution of 2-(propylamino)ethanol (4.60 g, 44.63 mmol) in THF (40 mL) at -10
°C. The mixture was stirred for 20 min at -10 °C, then treated with aqueous HCl (0.5 M, 40 mL) to
10 pH ~ 3 before being extracted with EtOAc. The combined organic layers were washed with brine,
dried with Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash
column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) to give 2-[bis(2-chloroethyl)
amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**26**) (3.73 g, 60%) as a yellow oil.
¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.38, 8.24 (2d, *J* = 2.8
15 Hz, 1H), 4.84, 4.80 (2t, *J* = 5.2 Hz, 1H), 3.72-3.65 (m, 5H), 3.63-3.42 (m, 4H), 3.39-3.31 (m, 2H),
3.24-3.12 (m, 2H), 1.66, 1.55 (2m, 2H), 0.94, 0.73 (2t, *J* = 7.4 Hz, 3H). HRMS(ESI) calcd for C₁₆H₂₃
Cl₂N₄O₆ [M+H]⁺ *m/z* 437.0989; found 437.0993.

A mixture of 2-[bis(2-chloroethyl) amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**26**)
20 (3.50 g, 8.00 mmol) and LiBr (13.90 g, 160.00 mmol) in MEK (70 mL) was heated under reflux with
stirring for 3 h, then concentrated under reduced pressure to dryness. The residue was dissolved in
EtOAc, washed with deionized water and then 10% aqueous NaBr before being dried with Na₂SO₄
and concentrated under reduced pressure. The residue was resubmitted to LiBr (13.90 g, 160.00
mmol) in MEK (70 mL) and then the above workup to provide the crude product, which was then
25 purified by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1) to give 2-
[bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**5**) (3.58 g, 85%) as a
yellow solid: m.p. (101-102 °C). ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.72, 8.69 (2d, *J* = 2.8
Hz, 1H), 8.39, 8.25 (2d, 2.8 Hz, 1H), 4.85, 4.82 (2t, *J* = 5.2 Hz, 1H), 3.69-3.52 (m, 8H), 3.48-3.31 (m,
4H), 3.24-3.16 (m, 2H), 1.66, 1.55 (2m, 2H), 0.93, 0.74 (2t, *J* = 7.3 Hz, 3H). Anal. Calcd for
30 C₁₆H₂₂Br₂N₄O₆·0.1EtOAc: C, 36.8; H, 4.3; N, 10.5; Br 29.9%; found: C, 37.2; H, 4.2; N, 10.7; Br
30.0%.

1.1.1.5 2-[Bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (7)

A stirred solution of 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**22**) (1.50 g, 4.26 mmol) in SOCl₂ (15 mL) and DMF (3 drops) was heated under reflux for 3 h, then cooled to room temperature and the excess SOCl₂ was removed under reduced pressure. The resulting crude 2-[bis(2-chloroethyl)amino]-3,5-dinitrobenzoyl chloride was dissolved in THF (12 mL) and slowly added to a stirred solution of 3-(methylamino)propanol (prepared according to the literature procedure; J.Org.Chem. 1979, 44, 2718) (1.14 g, 12.78 mmol) in THF (12 mL) at -10 °C. The mixture was stirred for 20 min at -10 °C, then treated with aqueous HCl (0.5 M, 12 mL) to pH ~ 3 before being extracted with EtOAc. The combined organic layers were washed with brine, dried with Na₂SO₄, concentrated under reduced pressure and the residue was recrystallized from CH₂Cl₂/hexane to give 2-[bis(2-chloroethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (**27**) (1.30 g, 72%) as a yellow solid: m.p. 125-128 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70 (d, *J* = 2.8 Hz, 1H), 8.28 (2d, *J* = 2.8, 1.0 Hz, 1H), 4.55, 4.38 (2t, *J* = 5.1 Hz, 1H), 3.71-3.67 (m, 4H), 3.54-3.44 (m, 5H), 3.36-3.26 (m, 3H), 3.00, 2.90 (2s, 3H), 1.81-1.74 (m, 1H), 1.70-1.66 (m, 1H). Anal. Calcd for C₁₅H₂₀Cl₂N₄O₆: C, 42.6; H, 4.8; N, 13.2%; found: C, 42.7; H, 4.8; N, 12.9%.

A mixture of 2-[bis(2-chloroethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (**27**) (1.30 g, 3.07 mmol) and LiBr (5.34 g, 61.52 mmol) in MEK (10 mL) was heated under reflux with stirring for 3 h, then concentrated under reduced pressure to dryness. The residue was dissolved in EtOAc, washed with deionized water and then 10% aqueous NaBr before being dried with Na₂SO₄ and concentrated under reduced pressure. The residue was resubmitted to LiBr (5.34 g, 61.52 mmol) in MEK (10 mL) before being worked up as above. Purification of the crude product by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1) provided 2-[bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (**7**) (1.21 g, 77%) as a yellow solid: m.p. > 300 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71 (d, *J* = 2.7 Hz, 1H), 8.29 (2d, 2.7, 1.4 Hz, 1H), 4.55, 4.37 (2t, *J* = 5.1 Hz, 1H), 3.54-3.50 (m, 9H), 3.41-3.31 (m, 3H), 3.01, 2.91 (2s, 3H), 1.81-1.75 (m, 1H), 1.71-1.64 (m, 1H). Anal. Calcd for C₁₅H₂₀Br₂N₄O₆: C, 35.2; H, 3.9; N, 10.9%; found: C, 35.5; H, 4.0; N, 10.7%.

30 1.1.1.6 2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (**9**)

A mixture of 2-[bis(2-chloroethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (**23**) (1.50 g, 3.67 mmol) and NaI (2.20 g, 14.66 mmol) in EtOAc (20 mL) was heated at 60 °C with stirring for 3 h, before being washed with deionized water, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was resubmitted to NaI (2.20 g, 14.66 mmol) in EtOAc (20

mL) before being worked up as above. Purification of the crude product by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1) provided 2-[bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (**9**) (1.35 g, 62%) as a yellow solid: m.p. 129-131 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.38, 8.33 (2d, *J* = 2.8 Hz, 1H), 4.86 (br, s, 1H), 3.68-3.12 (m, 12H), 2.95, 3.04 (2s, 3). Anal. Calcd for C₁₄H₁₈I₂N₄O₆: C, 28.4; H, 3.1; N, 9.5; I, 42.9%; found: C, 28.7; H, 3.0; N, 9.2; I, 42.6%.

1.1.1.7 2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**10**)

- 10 A mixture of 2-[bis(2-chloroethyl) amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**26**) (1.40 g, 3.20 mmol) and NaI (1.92 g, 12.8 mmol) in EtOAc (20 mL) was heated at 60 °C with stirring for 3 h, before being washed with deionized water, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was resubmitted to NaI (1.92 g, 12.8 mmol) in EtOAc (20 mL) before being worked up as above. Purification of the crude product by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (9:1), followed by trituration from Et₂O/hexane provided 2-[bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**10**) (1.28 g, 64%) as a yellow solid: m.p. 127-129 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.38, 8.24 (2d, *J* = 2.8 Hz, 1H), 4.83, 4.82 (2t, *J* = 5.2 Hz, 1H), 3.70-3.15 (m, 14H), 1.72, 1.47 (2m, 2H), 0.94, 0.73 (2t, *J* = 7.4 Hz, 3H). Anal. Calcd for C₁₆H₂₂I₂N₄O₆·0.05Hexane: C, 31.7; H, 3.5; N, 9.0; I, 41.0%; found: C, 31.4; H, 3.7; N, 9.0; I, 40.6%.
- 20

1.1.1.8 2-((2-Bromoethyl)-2-{[(2-hydroxyethyl)(methyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**12**) (reference compound)

- 25 A solution of **2** (10.06 g, 20.2 mmol) in MeCN (75 mL) was treated with AgOMs (3.69 g, 18.2 mmol) under reflux with stirring for 1 h. The mixture was concentrated under reduced pressure and the residue was extracted with EtOAc, evaporated, and chromatographed on silica gel. Elution with EtOAc/petroleum ether (2:1) gave unreacted **2** (3.59 g), while elution with EtOAc, concentration of the eluate to small volume and dilution with petroleum ether gave 2-((2-bromoethyl)-2-{[(2-hydroxyethyl)(methyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**12**) (3.48 g, 34%) as a yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.69 (2d, *J* = 2.8 Hz, 1 H), 8.40, 8.34 (2d, *J* = 2.8 Hz, 1 H), 4.87 (br s, 1 H), 4.31-4.19 (m, 2 H), 3.71-3.10 (m, 13 H), 3.03, 2.95 (2s, 3 H). HRMS(FAB) calcd for C₁₅H₂₂⁷⁹BrN₄O₉S [M+H]⁺ *m/z* 513.0219: found 513.0273.
- 30

1.1.1.9 2-(2-{{(2-Hydroxyethyl)(isopropyl)amino}carbonyl} {2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**19**)

A solution of *tert*-butyl 2-(bis{2-[(methylsulfonyl)oxy]ethyl}amino)-3,5-dinitrobenzoate (**42**) (Atwell et al, Journal of Labelled Compounds and Radiopharmaceuticals, 2007, 50, 7-12) (7.60 g, 14.4 mmol) in CH₂Cl₂ (60 mL) was cooled to 5 °C and treated with TFA (45 mL). The mixture was stirred at room temperature for 1.5 h, and then concentrated to dryness under reduced pressure. The residue was recrystallized from CH₂Cl₂/diisopropyl ether to give 2-(bis{2-[(methylsulfonyl)oxy]ethyl}amino)-3,5-dinitrobenzoic acid (**43**) as a yellow solid (6.79 g, 98%): m.p. 135-138 °C. ¹H NMR [(CD₃)₂SO] δ 14.11 (br, s, 1H), 8.82 (d, *J* = 2.8 Hz, 1H), 8.61 (d, *J* = 2.8 Hz, 1H), 4.29 (t, *J* = 5.5 Hz, 4H), 3.50 (t, *J* = 5.4 Hz, 4H), 3.12 (s, 6H). Anal. Calcd for C₁₃H₁₇N₃O₁₂S₂: C, 33.1; H, 3.6; N, 8.9%; found: C, 33.2; H, 3.6; N, 8.8%.

A mixture of 2-(bis{2-[(methylsulfonyl)oxy]ethyl}amino)-3,5-dinitrobenzoic acid (**43**) (600 mg, 1.27 mmol) and MgO (666 mg, 15.5 mmol) in CH₂Cl₂ (10 mL) was cooled to 0 °C and treated with oxalyl chloride (655 µL, 7.6 mmol) and DMF (5 drops). The mixture was stirred at 15 °C for 1 h, filtered and then treated with a solution of 2-(isopropylamino)ethanol (171 mg, 1.7 mmol) and triethyl amine (297 µL, 1.9 mmol) in CH₂Cl₂ (10 mL). The mixture was stirred at 0 °C for 30 min, diluted with CH₂Cl₂, washed with water and aqueous solution of methane sulfonic acid. The organic layers were dried with Na₂ SO₄ and then concentrated under reduced pressure. The residue was then purified by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) to provide 2-(2-{{(2-hydroxyethyl)(isopropyl)amino}carbonyl} {2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**19**) (470 mg, 66 %) as a yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.37, 8.26 (2d, *J* = 2.8 Hz, 1H), 4.81, 4.77 (2t, *J* = 5.7 Hz, 1H), 4.34-4.24 (m, 4H), 3.87-3.69 (2m, 1H), 3.63-3.31 (m, 7H), 3.26-3.17 (m, 1H), 3.15 (2s, 6H), 1.36 (d, *J* = 6.8 Hz, 3H), 1.21, 1.16 (2d, *J* = 6.5 Hz, 3H). Anal. Calcd for C₁₈H₂₈N₄O₁₂S₂: C, 38.8; H, 5.1; N, 10.1%; found: C, 39.2; H, 5.2; N, 9.8%.

1.1.1.10 2-(2-{{(2-Hydroxyethyl)(propyl)amino}carbonyl} {2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**20**)

A mixture of 2-(bis{2-[(methylsulfonyl)oxy]ethyl}amino)-3,5-dinitrobenzoic acid (**43**) (333 mg, 0.7 mmol) and MgO (341 mg, 8.5 mmol) in CH₂Cl₂ (5 mL) was cooled to 0 °C and treated with oxalyl chloride (364 µL, 4.2 mmol) and DMF (3 drops). The mixture was stirred at 15 °C for 1 h, filtered

and then treated with a solution of 2-(propylamino)ethanol (105 μ L, 0.9 mmol) and triethyl amine (160 μ L, 1.1 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred at 0 °C for 40 min, diluted with CH_2Cl_2 , washed with water and aqueous solution of methane sulfonic acid. The organic layers were dried with Na_2SO_4 and then concentrated under reduced pressure. The residue was then purified by flash column chromatography on silica gel eluting with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (99:1) to provide 2-(2- $\{[(2\text{-hydroxyethyl})(\text{propyl})\text{amino}]\text{carbonyl}\}\{2\text{-}[(\text{methylsulfonyl})\text{oxy}]\text{ethyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**20**) (230 mg, 58 %) as a yellow gum; ^1H NMR [$(\text{CD}_3)_2\text{SO}$] (mixture of rotamers) δ 8.71, 8.69 (2d, $J = 2.8$ Hz, 1H), 8.38, 8.25 (2d, $J = 2.8$ Hz, 1H), 4.85, 4.82 (2t, $J = 5.2$ Hz, 1H), 4.32-4.27 (m, 4H), 3.68-3.50 (m, 5H), 3.45-3.39 (m, 3H), 3.34-3.19 (m, 2H), 3.14 (2s, 6H), 1.70-1.60 (m, 2H), 0.93, 0.73 (2t, $J = 7.4$ Hz, 3H). HRMS(ESI) calcd for $\text{C}_{18}\text{H}_{29}\text{N}_4\text{O}_{12}\text{S}_2$ $[\text{M}+\text{H}]^+$ m/z 557.1218: found 557.1202.

1.1.1.11 2-((2-Bromoethyl)-2- $\{[(2\text{-hydroxyethyl})(\text{isopropyl})\text{amino}]\text{carbonyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**13**)

A mixture of 2-(2- $\{[(2\text{-hydroxyethyl})(\text{isopropyl})\text{amino}]\text{carbonyl}\}\{2\text{-}[(\text{methylsulfonyl})\text{oxy}]\text{ethyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**19**) (230 mg, 0.4 mmol) and LiBr (68 mg, 0.8 mmol) in EtOAc (25 mL) was heated with stirring at 45 °C for 3 h. The mixture was then cooled to the room temperature, diluted with EtOAc, washed with water, dried with Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel eluting with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (99:1) provided 2-((2-bromoethyl)-2- $\{[(2\text{-hydroxyethyl})(\text{isopropyl})\text{amino}]\text{carbonyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**13**) (110 mg, 49%) as a yellow gum; ^1H NMR [$(\text{CD}_3)_2\text{SO}$] (mixture of rotamers) δ 8.70, 8.68 (2d, $J = 2.8$ Hz, 1H), 8.37, 8.26 (2d, $J = 2.8$ Hz, 1H), 4.82, 4.76 (2t, $J = 5.8$ Hz, 1H), 4.32-4.15 (m, 2H), 3.87, 3.71 (2m, 1H), 3.63-3.33 (m, 8H), 3.30-3.18 (m, 2H), 3.14 (2s, 3H), 1.36 (d, $J = 6.8$ Hz, 3H), 1.20, 1.15 (2m, 3H).

1.1.1.12 2-((2-Bromoethyl)-2- $\{[(2\text{-hydroxyethyl})(\text{propyl})\text{amino}]\text{carbonyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**14**)

A mixture of 2-(2- $\{[(2\text{-hydroxyethyl})(\text{propyl})\text{amino}]\text{carbonyl}\}\{2\text{-}[(\text{methylsulfonyl})\text{oxy}]\text{ethyl}\}$ -4,6-dinitroanilino)ethyl methanesulfonate (**20**) (75 mg, 0.1 mmol) and LiBr (18 mg, 0.2 mmol) in EtOAc (15 mL) was heated with stirring at 45 °C for 1 h. The mixture was then cooled to the room temperature, diluted with EtOAc, washed with water, dried with Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel

eluting with CH₂Cl₂/MeOH (99:1) provided 2-((2-bromoethyl)-2-{[(2-hydroxyethyl)(propyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**14**) (33 mg, 45%) as a yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70, 8.69 (2d, *J* = 2.8 Hz, 1H), 8.38, 8.25 (2d, *J* = 2.8 Hz, 1H), 4.84, 4.82 (2t, *J* = 5.2 Hz, 1H), 4.31-4.26 (m, 2H), 3.70-3.32 (m, 10H), 3.25-3.16 (m, 2H), 3.14 (2s, 3H), 1.70-1.50 (m, 2H), 0.94, 0.73 (2t, *J* = 7.3 Hz, 3H). HRMS(ESI) calcd for C₁₇H₂₆BrN₄O₉S [M+H]⁺ *m/z* 541.0598: found 541.0603.

1.1.1.13 2-((2-Chloroethyl)-2-{[(2-hydroxyethyl)(isopropyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**16**)

A mixture of 2-(2-{[(2-hydroxyethyl)(isopropyl)amino]carbonyl} {2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**19**) (180 mg, 0.3 mmol) and LiCl (178 mg, 4.2 mmol) in EtOAc (25 mL) was heated with stirring at 60 °C for 5 h. The mixture was then cooled to the room temperature, diluted with EtOAc, washed with water and brine, dried with Na₂SO₄ and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) provided 2-((2-chloroethyl)-2-{[(2-hydroxyethyl)(isopropyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**16**) (86 mg, 54%) as a yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70, 8.68 (2d, *J* = 2.8 Hz, 1H), 8.37, 8.26 (2d, *J* = 2.8 Hz, 1H), 4.81, 4.76 (2t, *J* = 5.8 Hz, 1H), 4.32-4.15 (m, 2H), 3.87-3.65 (m, 3H), 3.63-3.32 (m, 7H), 3.27-3.17 (m, 1H), 3.14 (2s, 3H), 1.35 (d, *J* = 6.8 Hz, 3H), 1.20, 1.15 (2m, 3H). HRMS(ESI) calcd for C₁₇H₂₆ClN₄O₉S [M+H]⁺ *m/z* 497.1104: found 497.1107.

1.1.1.14 2-((2-Chloroethyl)-2-{[(2-hydroxyethyl)(propyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**17**)

A mixture of 2-(2-{[(2-hydroxyethyl)(propyl)amino]carbonyl} {2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**20**) (120 mg, 0.2 mmol) and LiCl (119 mg, 2.8 mmol) in EtOAc (20 mL) was heated with stirring at 60 °C for 5 h. The mixture was then cooled to the room temperature, diluted with EtOAc, washed with water and brine, dried with Na₂SO₄ and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel eluting with CH₂Cl₂/MeOH (99:1) provided 2-((2-chloroethyl)-2-{[(2-hydroxyethyl)(propyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**17**) (50 mg, 47%) as a yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.70, 8.69 (2d, *J* = 2.8 Hz, 1H), 8.37, 8.25 (2d, *J* = 2.8 Hz, 1H), 4.84, 4.82 (2t, *J* = 5.2 Hz, 1H), 4.31-4.26 (m, 2H), 3.73-3.32 (m, 10H),

3.25-3.18 (m, 2H), 3.14 (2s, 3H), 1.69-1.51 (m, 2H), 0.93, 0.73 (2t, $J = 7.3$ Hz, 3H). HRMS(ESI) calcd for $C_{17}H_{26}ClN_4O_9S$ $[M+H]^+$ m/z 497.1104; found 497.1111.

1.1.2 Preparation of the N-alkyl carboxamide mustard phosphates

5

1.1.2.1 2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl dihydrogen phosphate (34)

10 A stirred solution of 2-[bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (2) (5.69 g, 11.42 mmol) in 1*H*-tetrazole (123 mL, 52.55 mmol; 3% w/w solution in MeCN) and DMF (10 mL) at 10 °C was treated with slow addition of di-*tert*-butyl diisopropylphosphoramidite (95%, 14.4 mL, 45.68 mmol) under N_2 . The mixture was stirred at room temperature for 3 h, then cooled to -15 °C and treated with slow addition of a solution of *m*-CPBA (50%, 20.0 g, 115.87 mmol) in CH_2Cl_2 (150 mL). After further stirring at room temperature
15 for 2 h the reaction mixture was then concentrated under reduce pressure and the residue was dissolved in EtOAc, washed with 10% aqueous $Na_2S_2O_5$, 5% aqueous $NaHCO_3$ and water before being dried with Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with hexane/EtOAc (1:1) to give 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl di(*tert*-butyl) phosphate (28) (5.70 g,
20 72%) as a yellow gum. 1H NMR [$(CD_3)_2SO$] (mixture of rotamers) δ 8.72, 8.70 (2d, $J = 2.8$ Hz, 1H), 8.34, 8.29 (2d, $J = 2.8$ Hz, 1H), 4.13-3.72 (m, 3H), 3.62-3.31 (m, 9H), 3.05, 2.96 (2s, 3H), 1.45, 1.40, 1.37 (3s, 18H). HRMS(FAB) Calc. for $C_{22}H_{36}^{79}Br_2N_4O_9P$ (MH^+) m/z 689.0587, found: 689.0582; Calc. for $C_{22}H_{36}^{79}Br^{81}BrN_4O_9P$ (MH^+) m/z 691.0566, found: 691.0575; Calc. for $C_{22}H_{36}^{81}Br_2N_4O_9P$ (MH^+) m/z 693.0546, found: 693.0562.

25

A stirred solution of 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl di(*tert*-butyl) phosphate (28) (5.70 g, 8.26 mmol) in CH_2Cl_2 (140 mL) was treated with TFA (140 mL) at room temperature with stirring for 1 h, then concentrated under reduced pressure to remove the excess TFA. The resulted yellow residue was dissolved in MeCN, filtered and concentrated to give
30 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl dihydrogen phosphate (34) (4.77 g, 100%) as a yellow solid: m.p. 93-96 °C. 1H NMR [$(CD_3)_2SO$] (mixture of rotamers) δ 8.72, 8.68 (2d, $J = 2.7$ Hz, 1H), 8.40, 8.30 (2d, $J = 2.7$ Hz, 1H), 4.10-3.69 (m, 3H), 3.60-3.33 (m, 9H), 3.05, 2.97 (2s, 3H), signals corresponding to $P(OH)_2$ protons not observed. HRMS(FAB) Calc. for $C_{14}H_{20}^{79}Br_2N_4O_9P$ (MH^+) m/z 576.9335, found: 576.9323; Calc. for $C_{14}H_{20}^{79}Br^{81}BrN_4O_9P$ (MH^+) m/z 578.9314, found: 578.9325; Calc. for $C_{14}H_{20}^{81}Br_2N_4O_9P$ (MH^+) m/z 580.9294, found: 580.9293.

35

1.1.2.2 2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (ethyl)amino]ethyl dihydrogen phosphate (35)

- 5 A stirred solution of 2-[bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (3) (1.0 g, 1.95 mmol) in 1*H*-tetrazole (21 mL, 8.98 mmol; 3% w/w solution in MeCN) and DMF (1.5 mL) at 10 °C was treated with slow addition of di-*tert*-butyl diisopropylphosphoramidite (95%, 2.5 mL, 7.92 mmol) under N₂. The mixture was stirred at room temperature for 3 h, then cooled to -
- 10 15 °C and treated with slow addition of a solution of *m*-CPBA (50%, 3.37 g, 19.53 mmol) in CH₂Cl₂ (25 mL). After a further 2 h stirring at room temperature the reaction mixture was concentrated under reduce pressure and the residue dissolved in CH₂Cl₂, washed with 10% aqueous Na₂S₂O₅, 5% aqueous NaHCO₃ and water before being dried with Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with hexane/EtOAc (1:1) to give 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (ethyl)amino]ethyl
- 15 di(*tert*-butyl) phosphate (29) as a yellow gum (1.21 g, 88%). ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.73, 8.70 (2d, *J* = 2.7 Hz, 1H), 8.32, 8.26 (2d, *J* = 2.8 Hz, 1H), 4.17-3.76 (m, 3H), 3.64-3.19 (m, 11H), 1.45, 1.40, 1.37 (3s, 18H), 1.20, 1.07 (2t, 7.1 Hz, 3H). HRMS(ESI) Calc. for C₂₃H₃₇⁷⁹Br₂N₄NaO₉P (MNa⁺) *m/z* 725.0564, found: 725.0557; Calc. for C₂₃H₃₇⁷⁹Br⁸¹BrN₄NaO₉P (MNa⁺) *m/z* 727.0539, found: 727.0541; Calc. for C₂₃H₃₇⁸¹Br₂N₄NaO₉P (MNa⁺) *m/z* 729.0524,
- 20 found: 729.0517.

- A stirred solution of 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (ethyl)amino]ethyl di(*tert*-butyl) phosphate (29) (1.20 g, 1.70 mmol) in CH₂Cl₂ (35 mL) was treated with TFA (35 mL) at room temperature with stirring for 1 h, then concentrated under reduced pressure to remove excess TFA.
- 25 The resulted yellow solid was dissolved in MeCN, filtered and concentrated to give 2-[{2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (ethyl)amino]ethyl dihydrogen phosphate (35) (875 mg, 87%) as a yellow solid: m.p. 165-168 °C. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 11.03 (s, br, 2H), 8.72, 8.68 (2d, *J* = 2.7 Hz, 1H), 8.35, 8.28 (2d, *J* = 2.8 Hz, 1H), 4.13-3.77 (m, 3H), 3.63-3.24 (m, 11H), 1.20, 1.08 (2t, 3H). HRMS(ESI) Calc. for C₁₅H₂₀⁷⁹Br₂N₄NaO₉P (MH⁺) *m/z* 588.9325, found:
- 30 588.9340; Calc. for C₁₅H₂₀⁷⁹Br⁸¹BrN₄NaO₉P (MH⁺) *m/z* 590.9306, found: 590.9321; Calc. for C₁₅H₂₀⁸¹Br₂N₄NaO₉P (MH⁺) *m/z* 592.9289, found: 592.9304.

1.1.2.3 2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl} (isopropyl)amino]ethyl dihydrogen phosphate (36)

A stirred solution of 2-[bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (**4**) (600 mg, 1.14 mmol) in 1*H*-tetrazole (12.2 mL, 5.25 mmol; 3% w/w solution in MeCN) and DMF (0.8 mL) at 10 °C was treated with slow addition of di-*tert*-butyl diisopropylphosphoramidite (95%, 1.5 mL, 4.56 mmol) under N₂. The mixture was stirred at room temperature for 3 h, then cooled to -15 °C and treated with slow addition of a solution of *m*-CPBA (50%, 2.00 g, 11.4 mmol) in CH₂Cl₂ (20 mL). After a further 2 h stirring at room temperature the reaction mixture was concentrated under reduce pressure and the residue dissolved in CH₂Cl₂, washed with 10% aqueous Na₂S₂O₅, 5% aqueous NaHCO₃ and water before being dried with Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with hexane/EtOAc (2:1) to give 2-[[2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl](isopropyl)amino]ethyl di(*tert*-butyl) phosphate (**30**) as a yellow gum (616 mg, 75%). ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.74, 8.69 (2d, *J* = 2.8 Hz, 1H), 8.32, 8.29 (2d, *J* = 2.8 Hz, 1H), 4.29, 4.14 (2m, 1H), 3.94, 3.80 (2m, 1H), 3.65-3.23 (m, 11H), 1.45, 1.43 (2s, 18H), 1.34 (d, *J* = 6.8 Hz, 3H), 1.20, 1.18 (2m, 3H).

A stirred solution of 2-[[2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl](isopropyl)amino]ethyl di(*tert*-butyl) phosphate (**30**) (610 mg, 1.01 mmol) in CH₂Cl₂ (15 mL) was treated with TFA (15 mL) at room temperature with stirring for 1 h, then concentrated under reduced pressure to remove excess TFA. The resulted yellow solid was dissolved in CH₂Cl₂/diisopropyl ether and precipitated with hexane to give 2-[[2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl](isopropyl)amino]ethyl dihydrogen phosphate (**36**) (489 mg, 95%) as a yellow gum. ¹HNMR [(CD₃)₂SO] (mixture of rotamers) δ 8.73, 8.66 (2d, *J* = 2.7 Hz, 1H), 8.32, 8.29 (2d, *J* = 2.8 Hz, 1H), 4.29-3.30 (m, 15H), 1.34 (d, *J* = 6.8 Hz, 3H), 1.20, 1.18 (2m, 3H).

1.1.2.4 2-[[2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl](methyl)amino]ethyl dihydrogen phosphate (**37**)

A stirred solution of 2-[bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (**9**) (3.25 g, 5.49 mmol) and 1*H*-tetrazole (615 mg, 8.78 mmol) in DMF (15 mL) at 0 °C was treated dropwise with di-*tert*-butyl diisopropylphosphoramidite (95%, 2.37 mL, 7.13 mmol). The mixture was warmed to room temperature for 2 h, then cooled to 0 °C and treated with a solution of 70% aqueous H₂O₂ (1.5 mL) in THF (2 mL). The mixture was warmed to 15 °C for 30 min, then poured into ice-water and extracted with EtOAc (2x). The combined organic phases were washed with 5% aqueous Na₂S₂O₅, water (3x), dried, and concentrated under reduced pressure below 30 °C. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether (1:1), and the

product was precipitated from a CH₂Cl₂ solution with hexane to give 2-[{2-[bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl di(*tert*-butyl) phosphate (**31**) (2.92 g, 68%) as an unstable yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.8 Hz, 1 H), 8.32, 8.26 (2d, *J* = 2.8 Hz, 1 H), 4.21-4.03, 3.88-3.20 (2m, 12H), 3.05-2.96 (2s, 3 H), 1.45, 1.40, 1.37 (3s, 18 H).

5 HRMS(FAB) Calc. for C₂₂H₃₆I₂N₄O₉P [M+H]⁺ *m/z* 785.0310; found 785.0321.

A solution of 2-[{2-[bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl di(*tert*-butyl) phosphate (**31**) (1.22 g, 1.56 mmol) in CH₂Cl₂ (10 mL) was treated with TFA (10 mL) for 1 h at room temperature, then concentrated under reduced pressure below 30 °C to a small volume. The solution was diluted with CH₂Cl₂ (4 mL), filtered, then iPr₂O was slowly added to precipitate 2-[{2-[bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl dihydrogen phosphate (**37**) (0.96 g, 96%) as a yellow solid: mp 78-82 °C; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.66 (2d, *J* = 2.8 Hz, 1 H), 8.39, 8.28 (2d, *J* = 2.8 Hz, 1 H), 4.18-3.17 (m, 12 H), 3.05, 2.97 (2s, 3 H), P(OH)₂ signals not seen. HRMS(FAB) Calc. for C₁₄H₂₀I₂N₄O₉P [M+H]⁺ *m/z* 672.9058; found 672.9058.

1.1.2.5 2-[{2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(propyl)amino]ethyl dihydrogen phosphate (**38**)

A stirred solution of 2-[bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (**10**) (3.40 g, 5.48 mmol) and 1*H*-tetrazole (615 mg, 8.78 mmol) in DMF (15 mL) at 0 °C was treated dropwise with di-*tert*-butyl diisopropylphosphoramidite (95%, 2.37 mL, 7.13 mmol). The mixture was warmed to room temperature for 2 h, then cooled to 0 °C and treated with a solution of 70% aqueous H₂O₂ (1.5 mL) in THF (2 mL). The mixture was warmed to 15 °C for 30 min, then poured into ice-water and extracted with EtOAc (2x). The combined organic phases were washed with 5% aqueous Na₂S₂O₅, water (3x), dried, and concentrated under reduced pressure below 30 °C. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether (1:1), and the product was precipitated from a CH₂Cl₂ solution with hexane to give 2-[{2-[bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(isopropyl)amino]ethyl di(*tert*-butyl) phosphate (**32**) (2.73 g, 61%) as an unstable yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.71, 8.68 (2d, *J* = 2.7 Hz, 1 H), 8.26, 8.25 (2d, *J* = 2.7 Hz, 1 H), 4.21-3.97, 3.88-3.06 (2m, 14H), 1.75-1.44 (m, 2 H), 1.45, 1.39, 1.36 (3s, 18 H), 0.94, 0.74 (2t, *J* = 7.3 Hz, 3 H). HRMS(FAB) Calc. for C₂₄H₄₀I₂N₄O₉P [M+H]⁺ *m/z* 813.0623; found 813.0628.

A solution of 2-[{2-[bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(isopropyl)amino]ethyl di(*tert*-butyl) phosphate (**32**) (1.46 g, 1.80 mmol) in CH₂Cl₂ (12 mL) was treated with TFA (12 mL) for 1 h at

room temperature, then evaporated under reduced pressure below 30 °C. The residue was crystallised from TFA/*i*Pr₂O and then from Me₂CO/CH₂Cl₂/*i*Pr₂O to give 2-[(2-bis(2-iodoethyl)amino)-3,5-dinitrobenzoyl](propyl)amino]ethyl dihydrogen phosphate (**38**) (1.03 g, 82%) as a yellow solid: mp 139-140 °C; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 11.1 (br, 2 H), 8.71, 8.66 (2d, *J* = 2.7 Hz, 1 H), 8.29, 8.25 (2d, *J* = 2.7 Hz, 1 H), 4.16-2.90 (m, 14 H), 1.75-1.46 (m, 2 H), 0.94, 0.74 (2t, *J* = 7.3 Hz, 3 H). HRMS(FAB) Calc. for C₁₆H₂₄I₂N₄O₉P [M+H]⁺ *m/z* 700.9371; found 700.9366.

1.1.2.6 2-[(2-Bromoethyl)-2-({methyl[2-(phosphonooxy)ethyl]amino}carbonyl)-4,6-dinitroanilino]ethyl methanesulfonate (**39**) (reference compound)

A stirred solution of 2-[(2-bromoethyl)-2-({(2-hydroxyethyl)(methyl)amino}carbonyl)-4,6-dinitroanilino]ethyl methanesulfonate (**12**) (2.00 g, 3.90 mmol) in DMF (5 mL) at 10 °C was treated with 1*H*-tetrazole (17.3 mL, 5.85 mmol; 3% w/w solution in MeCN), followed by the slow addition of di-*tert*-butyl diisopropylphosphoramidite (95%, 1.68 mL, 5.06 mmol). The mixture was warmed to room temperature for 2 h, then cooled to 0 °C and treated with a solution of 70% aqueous H₂O₂ (0.9 mL) in THF (1 mL). The mixture was warmed to 15 °C for 20 min, then poured into ice-water and extracted with EtOAc (2x). The combined organic phases were washed with 5% aqueous Na₂S₂O₅, water (3x), dried, and concentrated under reduced pressure below 30 °C. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether (4:1). The eluate was concentrated to small volume and diluted with petroleum ether to give 2-[(2-bromoethyl)-2-(6-*tert*-butoxy-2,8,8-trimethyl-6-oxido-5,7-dioxa-2-aza-6-phosphanon-1-anoyl)-4,6-dinitroanilino]ethyl methanesulfonate (**33**) (1.96 g, 71%) as an unstable yellow gum; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.72, 8.70 (2d, *J* = 2.7 Hz, 1 H), 8.34, 8.29 (2d, *J* = 2.7 Hz, 1 H), 4.33-4.23, 4.19-4.04, 3.89-3.30 (3m, 12H), 3.16, 3.14 (2s, 3 H), 3.06, 2.97 (2s, 3 H), 1.44, 1.40, 1.37 (3s, 18 H). HRMS(FAB) Calc. for C₂₃H₃₉⁷⁹BrN₄O₁₂PS [M+H]⁺ *m/z* 705.1206; found 705.2111.

A stirred solution of 2-[(2-bromoethyl)-2-(6-*tert*-butoxy-2,8,8-trimethyl-6-oxido-5,7-dioxa-2-aza-6-phosphanon-1-anoyl)-4,6-dinitroanilino]ethyl methanesulfonate (**33**) (1.56 g, 2.21 mmol) in CH₂Cl₂ (8 mL) was treated with TFA (8 mL) for 1 h at room temperature, then concentrated under reduced pressure below 30 °C. The resulting gum was dissolved in CH₂Cl₂ (30 mL) and the filtered solution was refrigerated at 5 °C for 16 h. The separated yellow solid was collected, washed with CH₂Cl₂, and dried under high vacuum to give 2-[(2-bromoethyl)-2-({methyl[2-(phosphonooxy)ethyl]amino}carbonyl)-4,6-dinitroanilino]ethyl methanesulfonate (**39**) (1.16 g, 88%): mp 137-138 °C; ¹H NMR [(CD₃)₂SO] (mixture of rotamers) δ 8.72, 8.68 (2d, *J* = 2.8 Hz, 1 H), 8.41,

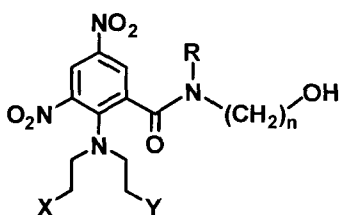
8.31 (2d, $J = 2.8$ Hz, 1 H), 4.35-4.20 (m, 2 H), 4.18-3.27 (m, 10 H), 3.15, 3.14 (2s, 3 H), 3.05-2.97 (2s, 3 H), $P(OH)_2$ signals not seen. HRMS(FAB) Calc. for $C_{15}H_{22}^{79}BrN_4O_{12}PS$ $[M+H]^+ m/z$ 592.9954; found 592.9953. Anal. Calcd for $C_{15}H_{22}BrN_4O_{12}PS$: C, 30.4; H, 3.7; N, 9.4; P, 5.2%; found: C, 30.5; H, 3.6; N, 9.3; P, 5.3%.

5

2. CYTOTOXICITY TESTING OF COMPOUNDS OF THE INVENTION

2.1 Optimisation of bystander cell killing

10 A series of dinitrobenzamide mustard prodrugs were synthesised and characterised by HPLC, MS, NMR and elemental analysis. Solubility and stability of compounds in media (+ 5% Fetal calf serum) were determined by HPLC. LogP values (n-octanol/water) were measured across a range of DNBM's and used to train the program ACD/LogPv9.0 with System Training and Accuracy
15 for the other compounds. A subset of representative compounds is provided in Table 1 in accordance with the generic structure provided.



20

Table 1:

Cmpd	X/Y	R	n	MW	LogP	Hydrogen bond	
						Donors	Acceptors
1	Br	H	2	484	2.3	2	10
2	Br	CH ₃	2	498	2.8	1	9
3	Br	C ₂ H ₅	2	512	3.3	1	9
4	Br	iPr	2	526	3.7	1	9
5	Br	nPr	2	526	3.9	1	9
6	Br	H	3	498	2.5	2	10
7	Br	CH ₃	3	512	3.0	1	9
8	I	H	2	578	2.9	2	10
9	I	CH ₃	2	592	3.4	1	9
10	I	nPr	2	620	4.4	1	9
11	Br/OSO ₂ CH ₃	H	2	499	1.0	2	13
12	Br/OSO ₂ CH ₃	CH ₃	2	513	1.6	1	12

- 5 In Table 1 above, compounds **1**, **6**, **8**, **11** and **12** are reference compounds, included to demonstrate the different, and advantageous, properties of the compounds of the invention.

Compounds were subjected to testing, including in a low-cell density cytotoxicity assay. Cells (500 cells/well) were seeded in 96-well plates and exposed to prodrug for 4h, washed, then left to grow for a further 5 days. The sulforhodamine B colourimetric assay was employed to measure cellular cytotoxicity and determine IC₅₀ values (concentration of prodrug required to inhibit proliferation by 50%). Parental HCT116 cells and cells expressing the illustrative nitroreductase gene NfsB from Escherichia coli (termed NTR) were evaluated head-to-head.

- 15 In parallel, a three dimensional high-cell density multi-cellular layer (MCL) clonogenic assay was performed. 1×10^6 cells of either 100% HCT-116^{WT} or containing 99% WT with a minor 1% HCT-116^{NTR} cell population were seeded into a collagen-coated Teflon microporous membrane and left to grow for 3 days. MCLs were then exposed to prodrug for 5h. After treatment, MCLs were enzyme dissociated, diluted in fresh medium and plated to determine clonogenic survival. To discriminate clonogenic activator (NTR+) from target (NTR-) colonies, cells were plated in non-selective medium (total cells) and medium containing 1 μ M puromycin (activator cells). Colonies were grown for 10 days before staining. Colonies containing >50 cells were counted.

- 25 The prodrug exposure concentration resulting in 90% cell kill (C₁₀; 10% survival) of NTR-naïve parental (WT) cells grown alone (C₁₀ = T), or when grown in intimate contact (co-culture) with 1%

NTR activators ($C_{10} = Tc$) was measured. The survival of the 1% NTR-expressing 'activators' themselves in co-culture ($C_{10} = Ac$) were also determined.

The low cell density and high cell density cytotoxicity results are summarised in **Table 2**:

5

Cmpd #	2D monolayer				3D multicellular layer		
	HCT116 ^{WT} IC ₅₀	HCT116 ^{NTR} IC ₅₀	WT:NTR	IC ₅₀	HCT116 ^{NTR} (Tc) C ₁₀	HCT116 ^{NTR} (Ac) C ₁₀	WT:NTR
	(uM)	(uM)	ratio		(uM)	(uM)	(Ac/Tc) C ₁₀ ratio
1	113	0.065	1738		127	0.13	991
2	115	0.24	479		133	0.12	1112
3	131	0.36	364		120	0.25	500
4	108	0.79	137		n/d	n/d	n/d
5	98	0.7	140		185	0.34	725
6	84	0.14	600		87	0.14	632
7	125	0.37	338		317	0.24	776
8	46	0.22	209		130	0.18	746
9	44	0.39	113		170	0.23	735
10	30	1.4	21		500	1.50	332
11	75	0.05	1500		110	0.04	2664
12	103	0.06	1717		158	0.05	3192

These data were subject to analysis to examine the relationship between physicochemical properties and cytotoxicity at low and high cell densities.

10

Figure 1 shows the correlation between lipophilicity (LogP) and cytotoxicity (IC₅₀) at low cell density.

It was observed that increasing prodrug lipophilicity (logP) was strongly associated with loss of potency against NTR-expressing HCT116 cells in 2D cell cultures ($r^2 = 0.92$; $p < 0.01$).

15

Figure 2 shows the correlation between lipophilicity (LogP) and cytotoxicity (C₁₀) against NTR-expressing 'activator' cells at high cell densities.

It was observed that increasing prodrug lipophilicity (logP) was strongly associated with loss of potency against NTR-expressing HCT116 cells in 3D cell cultures ($r^2 = 0.91$; $p < 0.01$).

20

Figure 3 shows the correlation between lipophilicity (LogP) and cytotoxicity (C₁₀) against NTR-naive 'target' cells when grown at high cell densities in the presence of 1% NTR-positive 'activator' cells.

25

Clonogenic kill of the 99% NTR-negative bystander cells by the 1% NTR in 3D culture MCLs could not be predicted by prodrug lipophilicity ($r^2 = 0.06$) or potency against the NTR expressing cells themselves at either low or high cell density (Figures 1 and 2, respectively). Thus, there was no apparent trend to any known parameter.

5

For example, compounds **2** and **3** provide unexpected improvement in bystander (NTR negative) cell sterilisation, where only ~ 1 μ M of prodrug is required to sterilise 90% of bystander 'target' cells when in the presence of NTR activator cells. This activity cannot be predicted based upon any known physicochemical property such as logP, molecular weight, hydrogen bond donor/acceptor groups or mustard leaving group. Further, the unexpected improvement in bystander cell killing cannot not predicted based upon measured cytotoxicity against NTR positive cells, irrespective of whether this is measured in two or three dimensions as shown in Figures 1 and 2, respectively.

10

Conventional screening methods would typically identify the most cytotoxic prodrugs against NTR-positive cells in low-density monolayer proliferation assays, which as demonstrated will fail to identify the most active compounds against bystander cells. Thus compounds **2** and **3** are unexpectedly superior in their ability to sterilise cells in local proximity to NTR positive cells. This property is highly desirable to maximise activity of NTR-based therapeutic strategies.

15

20

2.2 Activation of prodrugs by aldo-keto reductase 1C3 (AKR1C3).

DNBM prodrug PR-104 (reference compound **11**) has been reported as activated under aerobic conditions by the human ketosteroid reductase AKR1C3 (ACC. No. NM-003739), in addition to
5 being activated under hypoxia (Patterson, A.V., Guise, C.P., Abbattista, M., van Leeuwen, W., Pullen, S.M., Ferry, D.M., Denny, W.A., Guilford, P., and Wilson, W.R. The bioreductive prodrug PR-104 is activated under aerobic conditions by human aldo-keto reductase 1C3 (prostaglandin F synthase). *Eur J Cancer Suppl.*, 2008, 6[12], 473) It is considered that this relative loss of selectivity
10 for hypoxic reductase dependent metabolism, and thus for hypoxic cells, may be an undesirable feature for some patients and in some circumstances. This is based on the observation that AKR1C3 is expressed in a range of normal tissues, some of which are likely to be sensitive to the actions of activated nitrogen mustards.

Figure 4 shows a human normal tissue microarray stained for AKR1C3 expression.

15 A monoclonal antibody selective for AKR1C3 protein was used to evaluate 33 normal tissues (duplicate cores) using standard immunohistochemical detection methods. Significant expression is seen in bladder, stomach, small intestine, stomach, colon, kidney, liver, pancreas and thymus. Low level staining was also seen in 5-10 % of bone marrow cells. These tissues are likely to be
20 hypersensitive to exposure to prodrugs that can be activated by this nitroreductase.

2.3 Cellular cytotoxicity determinations for parental HCT116 cell populations and a clonal population stably expressing aldo-keto reductase 1C3 (AKR1C3)

Table 3

Cmpd #	HCT116 ^{WT} IC ₅₀ value (μ M)	HCT116 ^{AKR1C3} IC ₅₀ value (μ M)	HCT116 AKR1C3:WT sensitivity ratio #
1	70 $\pm$ 10	8.0 $\pm$ 1.5	5.5 $\pm$ 0.8
2	100 $\pm$ 18	21 $\pm$ 10	4.3 $\pm$ 1.0
3	114 $\pm$ 24	20 $\pm$ 4.5	5.8 $\pm$ 0.8
4	92 $\pm$ 12	27 $\pm$ 1.0	2.2 $\pm$ 0.1
5	80 $\pm$ 10	50 $\pm$ 7.1	1.1 $\pm$ 0.2
6	55 $\pm$ 14	2.6 $\pm$ 0.5	17 $\pm$ 7.3
7	104 $\pm$ 8.0	8.5 $\pm$ 0.1	10 $\pm$ 1.9
8	39 $\pm$ 6.3	3.1 $\pm$ 0.01	6.6 $\pm$ 0.2
9	37 $\pm$ 5.0	4.2 $\pm$ 0.6	7.1 $\pm$ 0.9
10	29 $\pm$ 2.5	16 $\pm$ 0.2	1.6 $\pm$ 0.2
11	59 $\pm$ 4.5	2.5 $\pm$ 0.8	23 $\pm$ 5.5
12	93 $\pm$ 15	2.0	33.5

Intra-experimental IC₅₀ value ratio (Mean $\pm$ SD)

Parental HCT-116^{WT} cell (ATCC CCL-247) and HCT-116^{AKR1C3} cells engineered to express human aldo-keto reductase 1C3 (AKR1C3; NM_003739) were passaged as monolayers in minimal essential media (α MEM; Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 5% FBS, (GIBCO NZ Ltd, Auckland, New Zealand) without antibiotics for <3 months from frozen stocks confirmed to be mycoplasma free by PCR-ELISA (Roche Diagnostics Mannheim, Germany). Cells were removed from T75 flask (Becton Dickinson Biosciences, Bedford, MA) with 1 ml of warm 0.05% trypsin/EDTA (Invitrogen Corporation, Carlsbad, CA, USA), counted (Z2 Coulter Particle Count and cell Analyzer, Beckman Coulter, Fullerton, CA, USA) and diluted to give 500/well (in 100 μ l) and left to attach for at $\geq$ 4 hrs (37°C, humidified incubator, 5% CO₂). Frozen stocks of compounds 1-12 (in DMSO) was thawed and diluted to required concentration with α MEM. Aliquots were diluted 1:100 so organic solvent concentrations (DMSO) in the cell cultures did not exceed 1%. 50 μ l of diluted drug stock was added directly to the top-well, where solubility limitations required, 150 μ l of drug solution was added directly to aspirated wells. The drugs were then diluted along the plate in 3-fold serial dilutions using a 12-channel pipette (Biolab Limited, Albany, Auckland, New Zealand). Plates were returned to the 37°C, 5% CO₂ incubator and left for 4 hrs. After the incubation period cells were washed three times and plates were filled with 200 μ l of α MEM containing 5% FCS and 1% penicillin/streptomycin (Gibco, Invitrogen Corporation, Grand Island, NY, USA), and left for a further 5 days in a 37 °C, 5% CO₂ incubator. After five days cells were fixed by

adding 67µl of cold 40% trichloroacetic acid (*Merck KGaA, Darmstadt, Germany*) to each well, to give a final concentration of 10%. Plates were held at 4 °C for 1 hour. After 1 hr, plates were rinsed in tap water 3-4 times, with excess water being drained prior to staining. After the plates were rinsed, 50µl of 0.4% sulforhodamine B (SRB; *Sigma-Aldrich, St. Louis, MO, USA*) in 1% acetic acid (*Merck KGaA, Darmstadt, Germany*) was added to each well and left for 30 minutes in the dark. Plates were rinsed in 1% acetic acid and excess fluid was drained. The stain was solubilised by the addition of 100µl of 10mM unbuffered Tris (*AppliChem GmbH, Darmstadt, Germany*), left for 1hr in the dark shaking at 150 rpm (*Barnstead-Labline; Barnstead International, Dubuque, IO, USA*). Plates were then read on an ELx 808 Absorbance Microplate Reader (*Bio-Tek Instruments, Winooski, VT, USA*).

Wavelengths on the plate reader were set at 490nm for measurement filter and 450nm for reference filter (the reference filter was subtracted from the measurement filter to give final absorbance: 490-450). The data was used to calculate the IC₅₀ value (KC4 microplate data analysis software V3.4, Bio-Tek), where 50% suppression of cell growth has occurred relative to untreated controls. N = 2-6 independent experiments IC₅₀ value (Mean ± 1 SD) were determined for each HCT116 population and intra-experimental sensitivity ratio calculated (Mean ± 1 SD).

Analysis of compounds for their ability to inhibit the growth of HCT116 cancer cells engineered to express AKR1C3 (by stable expression vector transfer) has provided the unexpected finding that selected alkyl substituents on the nitrogen of the carboxyl side chain can ameliorate cellular sensitivity to prodrug candidates in air.

This is illustrated by the compound series **1** to **5**, where compounds **4** and **5** have lost their ability to inhibit the growth of AKR1C3 expressing cells over parental non-expressing cells. The loss of activity for compound **5** by addition of an N-propyl group is independently confirmed with compound **8** (NH) and **9** (N-methyl) versus **10** (N-propyl), where an identical structure activity relationship (SAR) is observed. This SAR is displayed in Figure 5.

Figure 5 shows cell cytotoxicity data that indicates incremental N-alkyl extension of C2 hydroxyalkyl carboxamide sidechains diminishes AKR1C3 dependent cell sensitivity under aerobic conditions.

Thus, compounds with such N-alkyl extensions are increasingly less susceptible to AKR1C3 activation. This is particularly notable with compounds of the invention where X and Y are the same, as well as with compounds of the invention where X and Y are different and with an extended alkyl chain (R = propyl onwards). This diminished susceptibility to aerobic AKR1C3-mediated activation restores selectivity for hypoxic reductase activation. It also reduces if not eliminates

unintended activation of the prodrugs of the invention where a GDEPT or other approach involving an exogenous nitroreductase is employed.

5 It is appreciated that variations and modifications may be made to the invention as described without departing from the spirit and scope of the invention.

Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprising” and the like, are construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of “including, but not limited to”.

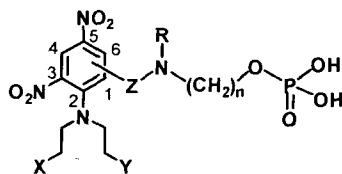
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The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that that prior art forms part of the common general knowledge.

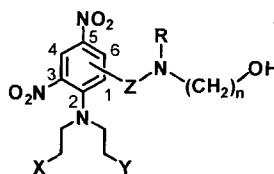
15 The foregoing describes the invention including preferred forms thereof. Alterations or modifications that would be apparent to the skilled person are intended to be included.

CLAIMS

1. A compound of Formula I or Formula II:



I



II

wherein

X represents Cl, Br, I, or OSO_2R_1 ,

Y represents Cl, Br, I, or OSO_2R_1 ,

Z represents at any available ring position $-\text{CO}-$ or $-\text{SO}_2-$,

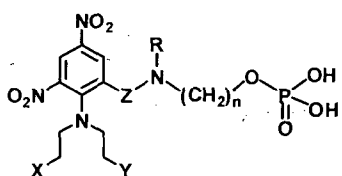
R represents: (1) a straight or branched chain C_{1-6} alkyl group or $-\text{CH}_2\text{CH}_2\text{OMe}$, when X and Y are the same, or (2) a straight or branched chain C_{3-6} alkyl group or $-\text{CH}_2\text{CH}_2\text{OMe}$ when X and Y are different,

R_1 represents a straight or branched chain C_{1-6} alkyl group, and

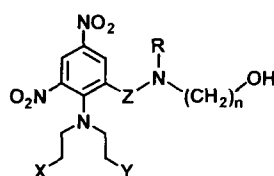
n is an integer from 1 to 6;

and pharmaceutically acceptable salts thereof.

2. A compound according to claim 1, of Formula Ia or IIa:



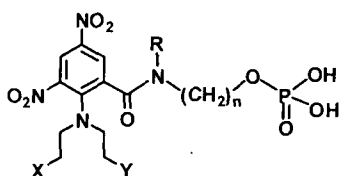
Ia



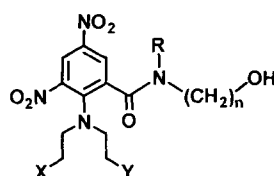
IIa

wherein X, Y, Z, R and n are as defined in claim 1.

3. A compound according to claim 1, of Formula Ib or IIb:



Ib



IIb

wherein X, Y, R and n are as defined in claim 1.

4. A compound of Formula Ib or IIb according to claim 3, where n is an integer of from 1 to 3.

5

5. A compound of Formula Ib or IIb according to claim 3 or 4, where X and Y are the same and R is selected from methyl, ethyl, propyl and isopropyl.

10

6. A compound of Formula Ib or IIb according to any one of claims 3 to 5, wherein X and Y are both Br.

7. A compound of Formula Ib or IIb according to any one of claims 3 to 5, wherein X and Y are both I.

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8. A compound of Formula Ib or IIb according to any one of claims 3 to 5, wherein X and Y are both OSO_2CH_3 .

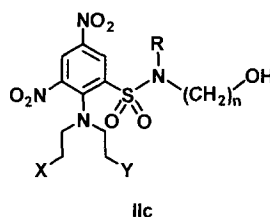
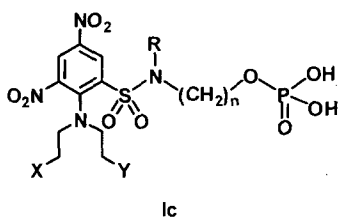
9. A compound of formula Ib or IIb according to claim 3 or 4, wherein X and Y are different and R is selected from propyl, isopropyl and butyl.

20

10. A compound of Formula Ib or IIb according to any one of claims 3, 4 and 9, wherein one of X and Y is Br or Cl and the other is OSO_2CH_3 .

11. A compound according to claim 1, of Formula Ic or IIc:

25



wherein X, Y, R and n are as defined for Formulae I and II.

12. A compound of Formula I according to claim 1, selected from the group consisting of:

30

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl dihydrogen phosphate (34),

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(ethyl)amino]ethyl dihydrogen phosphate (35),

2-[{2-[Bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl}(isopropyl)amino]ethyl dihydrogen phosphate (36),

2-[{2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(methyl)amino]ethyl dihydrogen phosphate (37) and

2-[{2-[Bis(2-iodoethyl)amino]-3,5-dinitrobenzoyl}(propyl)amino]ethyl dihydrogen phosphate (38);

and pharmaceutically acceptable salts thereof.

13. A compound of Formula II according to claim 1, selected from the group consisting of:

2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (2),

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (3),

2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-isopropyl-3,5-dinitrobenzamide (4),

2-[Bis(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (5),

2-[Bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-*N*-methyl-3,5-dinitrobenzamide (7),

2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-*N*-methyl-3,5-dinitrobenzamide (9),

2-[Bis(2-iodoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitro-*N*-propylbenzamide (10),

2-((2-Bromoethyl)-2-{[(2-hydroxyethyl)(isopropyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**13**),

2-((2-Bromoethyl)-2-{[(2-hydroxyethyl)(propyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**14**),

2-((2-Chloroethyl)-2-{[(2-hydroxyethyl)(isopropyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**16**),

2-((2-Chloroethyl)-2-{[(2-hydroxyethyl)(propyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (**17**),

2-(2-{[(2-Hydroxyethyl)(isopropyl)amino]carbonyl}{2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**19**),

2-(2-{[(2-Hydroxyethyl)(propyl)amino]carbonyl}{2-[(methylsulfonyl)oxy]ethyl}-4,6-dinitroanilino)ethyl methanesulfonate (**20**),

2-[Bis(2-bromoethyl)amino]-*N*-(4-hydroxybutyl)-*N*-methyl-3,5-dinitrobenzamide (**101**),

2-[Bis(2-bromoethyl)amino]-*N*-(5-hydroxypentyl)-*N*-methyl-3,5-dinitrobenzamide (**102**),

2-[Bis(2-bromoethyl)amino]-*N*-(6-hydroxyhexyl)-*N*-methyl-3,5-dinitrobenzamide (**103**),

2-[Bis(2-bromoethyl)amino]-*N*-(hydroxymethyl)-*N*-(3-hydroxypropyl)-3,5-dinitrobenzamide (**104**),

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(4-hydroxybutyl)-3,5-dinitrobenzamide (**105**),

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(5-hydroxypentyl)-3,5-dinitrobenzamide (**106**), and

2-[Bis(2-bromoethyl)amino]-*N*-ethyl-*N*-(6-hydroxyhexyl)-3,5-dinitrobenzamide (**107**);

and pharmaceutically acceptable salts thereof.

14. A pharmaceutical composition comprising a compound of Formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

5 15. A method for the production of an anti-cancer effect in a warm-blooded animal such as a human, wherein the method comprises administering to the animal an effective amount of a compound of Formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof.

10 16. A method for the production of an anti-cancer effect in a cell, wherein the method comprises contacting the cell with an effective amount of a compound of formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof.

15 17. The use of a compound of Formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof in the preparation of a medicament for the production of an anti-cancer effect in a warm-blooded animal such as a human.

20 18. A method for the treatment of a cancer in a warm-blooded animal such as a human, which comprises administering to the animal an effective amount of a compound of Formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof.

19. The use of a compound of Formula I or II as defined in claim 1 or a pharmaceutically acceptable salt thereof in the preparation of a medicament for the treatment of cancer.

25 20. A method according to any one of claims 15, 16 and 18, further including the step of administering radiation treatment.

30 21. A method of ablating tumour cells, comprising administering a compound of Formula I or Formula II as defined in claim 1, or a pharmaceutically acceptable salt thereof, to said cells in an amount effective to ablate those cells.

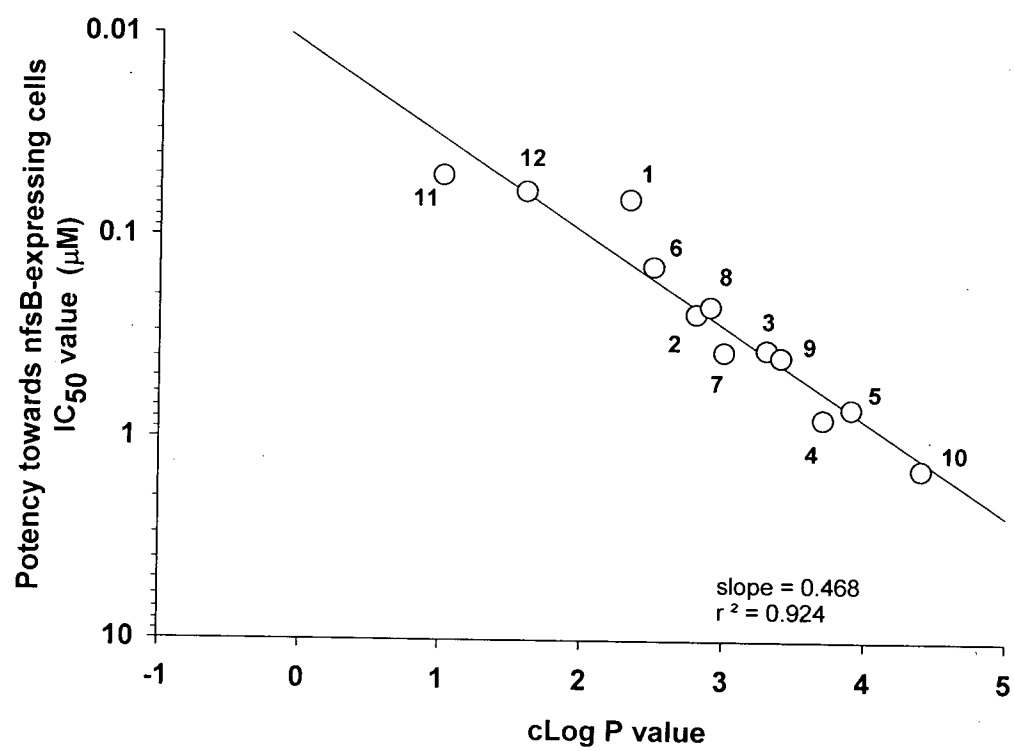
22. A method according to any one of claims 15, 16, 20 and 21, wherein the compound of Formula I or II is administered in combination with a therapy that results in expression of an exogenous nitroreductase enzyme within, or therapeutically proximate to, a tumour.

- 5 23. A method according to claim 22, wherein the expression of the nitroreductase enzyme results from GDEPT (gene-directed enzyme prodrug therapy) and the nitroreductase enzyme that is expressed is encoded by the *nfsB* gene of either *E. coli* or orthologous genes in *Clostridia* species.

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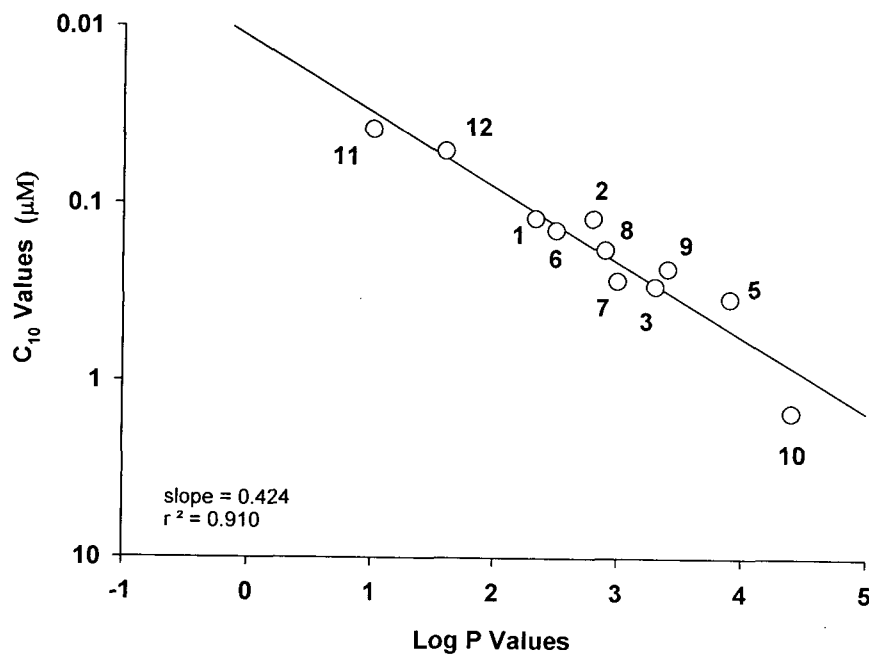
Figure 1



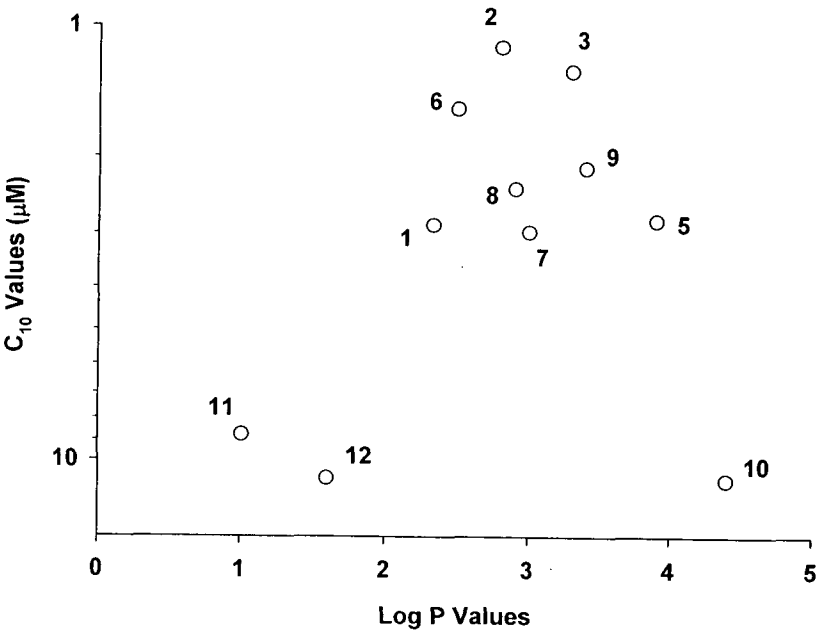
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Figure 2



5 Figure 3



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Figure 4

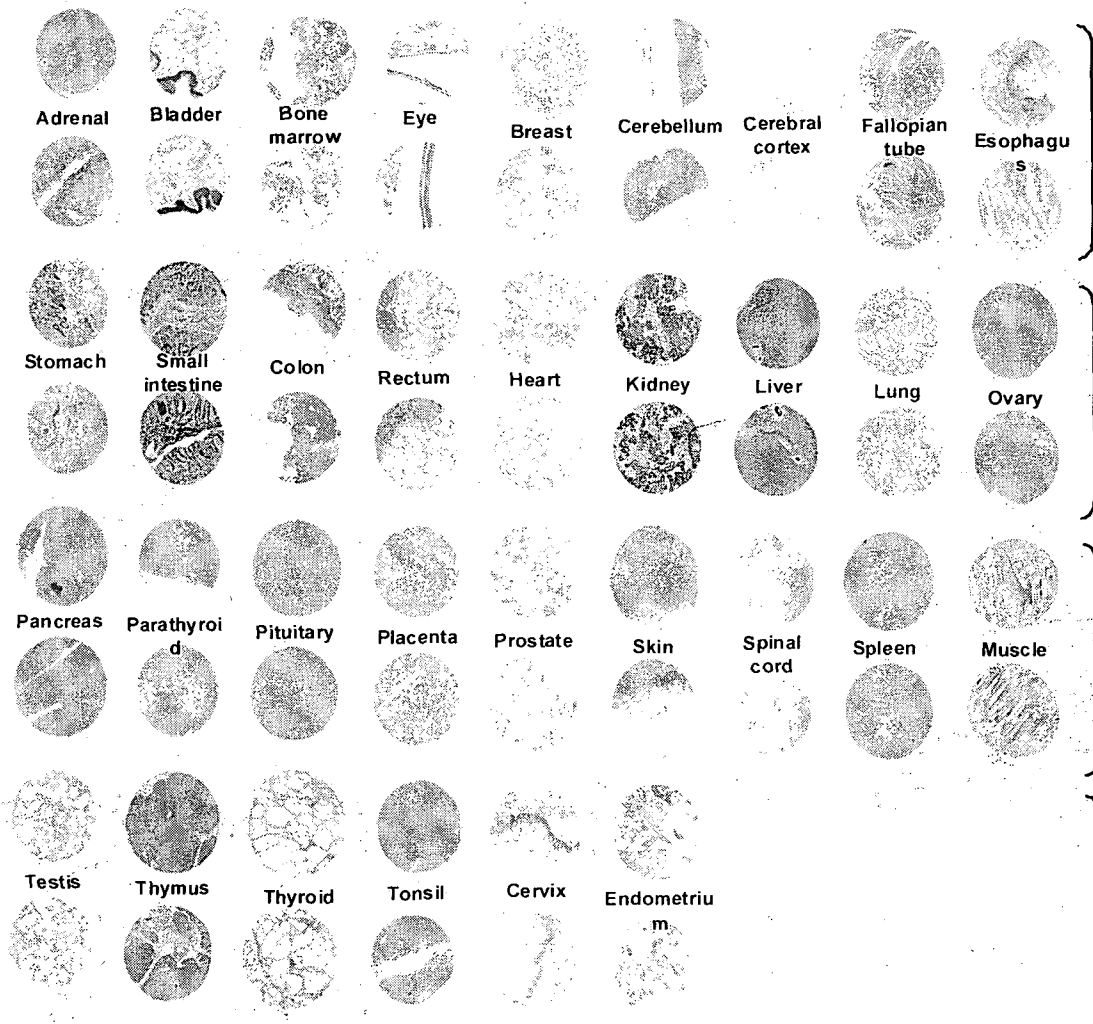
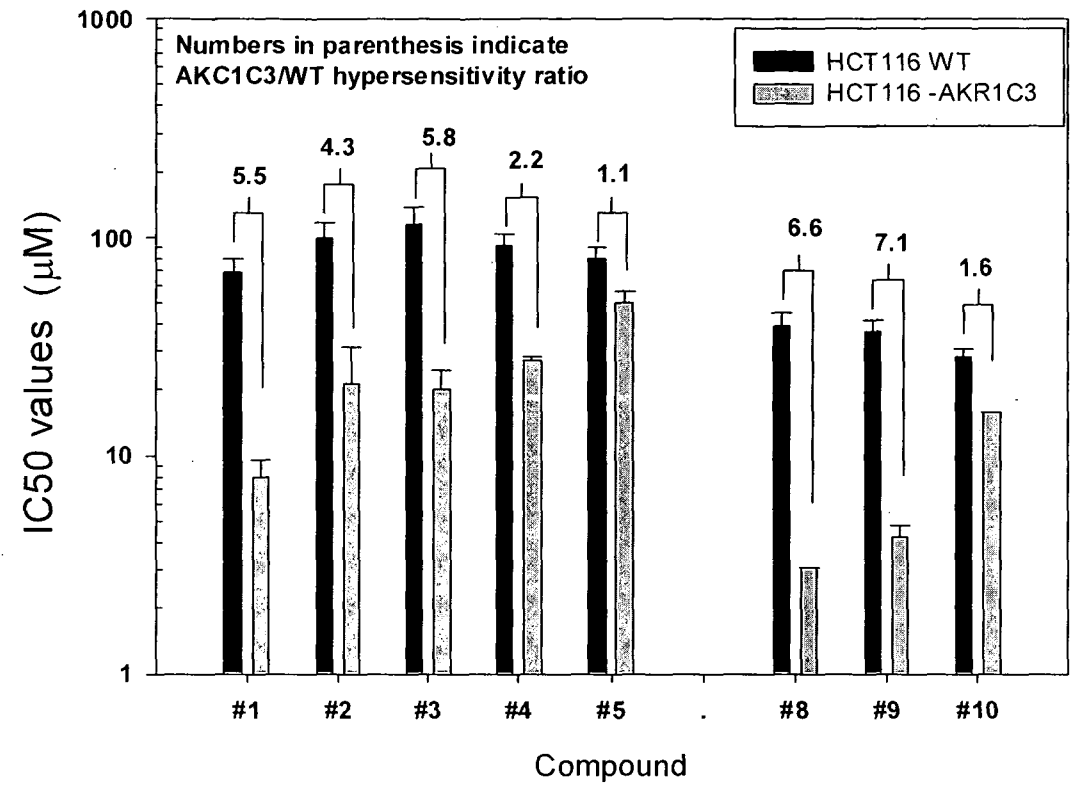


Figure 5



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ2009/000226

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. <i>C07C 233/69</i> (2006.01) <i>A61K 31/63</i> (2006.01) <i>C07C 311/17</i> (2006.01) <i>A61K 31/166</i> (2006.01) <i>A61K 31/661</i> (2006.01) <i>C07F 9/09</i> (2006.01) <i>A61K 31/18</i> (2006.01) <i>A61P 35/00</i> (2006.01) <i>A61K 31/255</i> (2006.01) <i>C07C 309/66</i> (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) STN CAS Online – File Registry and CAPlus: Structure search based on exemplified compounds		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1993/011099 A1 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD), 10 June 1993 See title; abstract; formula I in claim 1 and page 1 lines 9-12; page 35 lines 21-25 and claim 13; page 36 lines 8-10, 28-32 and page 37 lines 1-6; page 37 lines 2-6; page 38 lines 5-19; scheme 2 on page 4; scheme 4 on page 6	1-8, 11, 14-19, 21-22
Y	See title; abstract; formula I in claim 1 and page 1 lines 9-12; page 35 lines 21-25 and claim 13; page 36 lines 8-10, 28-32 and page 37 lines 1-6; page 37 lines 2-6; page 38 lines 5-19;	9-10, 20, 23
☒ 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 2 February 2010		Date of mailing of the international search report - 4 FEB 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. +61 2 6283 7999		Authorized officer Grace Wong AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2915

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ2009/000226

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/042471 A1 (AUCKLAND UNISERVICES LTD), 12 May 2005 See title; abstract; page 1 lines 5-10; claims 1 and 4; page 61 lines 1-6; page 17 lines 8-10 and claims 19-25 and 30; compounds IIa-12, IIa-13, IIb-2m on page 23	9-10, 20, 23
Y	WO 2008/030112 A1 (AUCKLAND UNISERVICES LTD), 13 March 2008 See title; page 10 lines 26-31; compounds 5 and 33b on page 28	9-10
Y	WO 2008/033040 A1 (AUCKLAND UNISERVICES LTD), 20 March 2008 See title; abstract; page 1 lines 5-6; compounds of claim 1; compounds of claims 3 and 4	9-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/NZ2009/000226

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			
WO	1993/011099	AU	29526/92	CA	2124315	EP	0616609
		NZ	240785	US	5571845	US	5750782
WO	2005/042471	AU	2004285831	BR	PI0416085	CA	2544335
		CN	1902159	EP	1680394	KR	20070006677
		NZ	529249	RU	2006118785	US	7629332
		US	2007032455	US	2010010094	ZA	200603105
WO	2008/030112	EP	2059499	NZ	549659		
WO	2008/033040	EP	2056839	NZ	549831	US	2008064665
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							