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**(54) Title:** EXPEDITIOUS SYNTHESIS OF DPD

**(57) Abstract:** This invention provides a practical synthesis route for 4,5-dihydroxypentane-2,3-dione (DPD), an unstable small molecule which is proposed to be the source of universal signaling agents for quorum sensing in bacteria. The synthesis route includes new intermediates and allows preparation of isotopically-labeled DPD and ent-DPD. The method provides sufficient quantities of DPD for study of spontaneous binding of borate to DPD, the signal for the marine bacteria *V. harveyi*

# EXPEDITIOUS SYNTHESIS OF DPD

**[0001]** This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Serial No. 60/611,551, filed September 20, 2004, which is incorporated herein by reference in its entirety.

## GOVERNMENT INTERESTS

**[0002]** This invention was funded in part through grants from the National Institutes of Health. Therefore, the federal government has certain rights in this invention.

## FIELD OF THE INVENTION

**[0003]** This invention provides a practical synthesis route for 4,5-dihydroxypentane-2,3-dione (DPD), a relatively unstable small molecule which is proposed to be the source of universal signaling agents for quorum sensing in bacteria. The synthesis route includes new intermediates and allows preparation of isotopically-labeled DPD and ent-DPD. The method provides sufficient quantities of DPD for study of spontaneous binding of borate to DPD, the signal for the marine bacteria *V. harveyi*.

## BACKGROUND OF THE INVENTION

**[0004]** DPD (**1**) is an enigmatic molecule. It has been known since 1971 as the product of catabolism of *S*-adenosylhomocysteine in many bacteria (Duerre *et al.* 1971); and, more recently, it is proposed to be the core molecule from which all bacterial AI-2 signaling molecules are derived (Miller *et al.* 2004). These molecules are widely used in inter-species communication in the bacterial world (Federle *et al.* 2001). It is a simple molecule, but was reported to be quite unstable toward rearrangement (Winzer *et al.* 2002; Slaughter *et al.* 1999) and oligomerization (Semmelhack *et al.* 2004; Meijler *et al.* 2004), and has only recently been synthesized and tentatively characterized in dilute solution (Meijler *et al.* 2004).

**[0005]** Fig. 1A shows that DPD can exist as an equilibrium mixture of three isomers (**1, 2**, and **3**), hydrated versions (**4** and **5**), and borate complexes (e.g., **6**), collectively known as autoinducer 2 (AI-2) (Miller *et al.* 2004). *V. harveyi* recognizes the 2,3-borate diester (**6**) of the hydrated α-anomer (**4**) of DPD as AI-2 (Chen *et al.*

2002). *S. typhimurium* recognizes the hydrated β-anomer (**5**) of DPD without borate (Miller *et al.* 2004). The incorporation of borate into the AI-2 signal may be a function of the natural habitat of the bacteria. *V. harvyei* is a marine bacterium that lives in an environment with high borate concentration compared to *S. typhimurium*, an enteric bacterium.

**[0006]** An efficient synthesis strategy for DPD allows characterization of DPD isomers, hydration processes, and boron complexation. The availability of much larger quantitites of DPD than previously obtainable (either enzymatically or by a less efficient synthetic route should facilitate understanding DPD's role in bacterial quorum sensing for many species.

## SUMMARY OF THE INVENTION

**[0007]** The present invention relates to an efficient and practical synthesis for DPD, its isomers and isotopically-labeled DPD. The availability of large quantities of DPD, as well as isotopically-labeled versions thereof, allows a detailed study of the isomerization, hydration, and boron complexation phenomena of DPD and aids in elucidation of DPD's role in bacterial quorum sensing.

**[0008]** One aspect of the invention relates to a method to synthesize protected DPD. Protected DPD can be purified and isolated as a solid, permitting storage of large quantities of this DPD precursor.

**[0009]** It has been discovered in accordance with this invention that DPD is stable under acidic conditions and that conversion of protected DPD to DPD proceeds efficiently and to near completion (as much as 95-99% removal of the protection group. Hence, another aspect of the invention takes advantage of this characteristic. Protected DPD is prepared in acid solutions and after hydroylsis of the protecting group, the pH is adjusted, and the solution used (directly or with dilution) in biological and other evaluations. For biological evaluations, the by-product of deprotection does not interfere with biological quorum sensing assays, at least when the protecting group is cyclohexylidene.

**[0010]** Yet another aspect of the invention relates to the novel chemical intermediates used in synthesis of DPD as well as the isotopically-labeled forms of DPD and *ent*-DPD, and the pharmaceutically acceptable salts, if relevant. These intermediates include compounds **10, 11** and **12** of the invention (Fig. 2A) and the

isotopically-labeled intermediates include compounds **15-18** as well as their $^{14}C$ labeled counterparts (Fig. 1B).

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0011]** Fig. 1A illustrates the biological forms of DPD (**1-6**).

**[0012]** Fig. 1B illustrates various DPD derivatives (**13-21**).

**[0013]** Fig 2A shows the synthesis strategy for DPD.

**[0014]** Fig 2B illustrates the chemical reactions for 1) converting **10** to **11**, and 2) the hydration step to obtain hydrated DPD (**4** or **5**). Hydrated DPD complexes (with borate) when present at pH > 7 to produce **6** and borated isomers.

**[0015]** Fig. 3 shows the comparative bioactivity of enzymatically-produced DPD, synthetic DPD and synthetic *ent*-DPD.

**[0016]** Fig. 4A shows the effects of low pH on enzymatically-produced DPD.

**[0017]** Fig. 4B shows the effects of low pH on synthetic DPD.

**[0018]** Fig. 5 shows $^{13}C$ NMR spectra (panels a-c) and $^{11}B$ NMR spectra (panels d-f) when 15 mM DPD is titrated with borate at pH 7.8. For the $^{13}C$ NMR titration: (a) no added $B(OH)_3$; (b) 45 mM $B(OH)_3$; (c) sat. $B(OH)_3$; for the $^{11}B$ NMR titration: (d) no added $B(OH)_3$; (e) 40 mM $B(OH)_3$; (f) sat. $B(OH)_3$.

## DETAILED DESCRIPTION OF THE INVENTION

### Synthetic Methods

**[0019]** For the synthesis of the protected DPD molecules, the method involves (a) reacting gluconic acid γ-lactone with dimethoxycyclohexanone for a time and under conditions to produce 5,6-*O*-cyclohexylideneglucono-1,4-lactone; (b) subjecting the lactone to oxidative cleavage to produce 2,3-*O*-cyclohexylideneglyceraldehyde; (c) halogenating the glyceraldehyde molecule under conditions to produce 1,1-dihalo-3,4-cyclohexylidenedioxybut-1-ene ("dihaloalkene"); (d) purifying the dihaloalkene before reacting converting its alkene moiety to an alkyne anion; (e) quenching the anion by one of two routes, by reacting the alkyne anion with i) a methylating agent to produce 1,1-dihalo-3,4-cyclohexylidenedioxypent-3-yne, or ii) with water to produce 1,1-dibromo-3,4-cyclohexylidenedioxybut-3-yne and subsequently methylating that compound to produce 1,1-dibromo-3,4-cyclohexylidenedioxypent-3-yne, and recovering that alkyne; (f) oxidizing that alkyne in non-aqueous solution under conditions to thereby

produce 4,5-cyclohexylidenedioxy-2,3-pentadione, which is the protected DPD molecule; and (g) recovering protected DPD.

**[0020]** When the gluconic acid γ-lactone starting material is a racemic mixture of the L- and D- stereoisomers of gluconic acid γ-lactone, then the protected DPD is a racemic mixture of (S)-4,5-cyclohexylidenedioxy-2,3-pentadione and (R)-4,5-cyclohexylidenedioxy-2,3-pentadione (racemic DPD). The present method also allows stereospecific synthesis of DPD and ent-DPD. When the gluconic acid γ-lactone starting material is L-gluconic acid γ-lactone, the protected DPD is (S)-4,5-cyclohexylidenedioxy-2,3-pentadione (DPD, **1**). When the gluconic acid γ-lactone starting material is D-gluconic acid γ-lactone, the protected DPD is (R)-4,5-cyclohexylidenedioxy-2,3-pentadione (ent-DPD, **14**).

**[0021]** For step (a), the reaction of gluconic acid γ-lactone with dimethoxycyclohexanone to produce 5,6-*O*-cyclohexylideneglucono-1,4-lactone is preferably done according to the method of Vekemans *et al.* 1985. Other methods known to those of skill in the art can be used and those giving a high yield of the lactone are preferred.

**[0022]** The lactone can be used directly without further purification in step (b) or can be purified before being subjected to oxidative cleavage to produce 2,3-*O*-cyclohexylideneglyceraldehyde. The oxidative cleavage preferably uses $KIO_4$ as described in Example 3. These conditions can be varied as known by those of skill in the art. Moreover, other oxidation methods that lead to the glyceraldehyde can be used.

**[0023]** For step (c), the glyceraldehyde is halogenated to produce 1,1-dihalo-3,4-cyclohexylidenedioxybut-1-ene. Halogenation can be accomplished using fluorine, chlorine, bromine or iodine and halogenation methods are well known in the art. The preferred halogenation method is bromination according to the method of Yoshida *et al.* 1992), see Example 4, or other variation of the Corey-Fuchs protocol. If necessary, the dihaloalkene is purified before further use, and this is preferable for the dibromoalkene.

**[0024]** In step (d), the alkene moiety of the dihaloalkene is converted to an alkyne anion with concomitant loss of the halogens. The alkyne anion can be quenched in step (e) to produce the alkyne by either of two routes and it is this point in the synthesis that allows introduction of carbon isotopes $^{13}C$ or $^{14}C$.

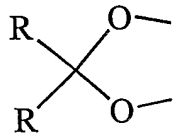
**[0025]** By one route, the alkyne anion is methylated to produce 1,1-dihalo-3,4-cyclohexylidenedioxypent-3-yne. Methylation reagents and conditions are well known and isotopically substituted methylation agents, such as $^{13}CH_3I$ and $^{14}CH_3I$ are readily obtainable. The alkyne should be purified before further use in the synthesis, but this may be optional under some conditions.

**[0026]** By the other route, the anion is quenched with water to produce 1,1-dibromo-3,4-cyclohexylidenedioxybut-3-yne and that molecule is subsequently methylated to produce 1,1-dibromo-3,4-cyclohexylidenedioxypent-3-yne. The methylation step of this route can be done as described in the paragraph above, see also, *e.g.*, Example 5. Quenching with water before methylation appears to improve the yield of the alkyne. This route is also useful for isotopically labeling the compounds, see, *e.g.*, Example 7. The alkyne should be purified before further use in the overall synthesis, but this may be optional under some conditions.

**[0027]** The next step, (f), involves oxidizing the alkyne in non-aqueous solution to produces 4,5-cyclohexylidenedioxy-2,3-pentadione (*i.e.*, the protected DPD molecule). The preferred oxidation protocol uses $RuO_2/NaIO_4$ and produces the protected DPD is high yield that can be readily purified. This method is that of Seebach (Zibuch *et al.* 1988) but other oxidation methods are suitable. The method of Seebach provides excellent yields.

**[0028]** The Examples provide details of these syntheses steps and those of skill in the art know how to vary solvents, temperature and reactions conditions to achieve these synthetic steps. Methods of purifying the various intermediates and compounds described herein are also well known to those of skill in the art. The examples illustrate, but should not be considered limiting to, a variety of purification schemes.

**[0029]** In a variation on the above method, the synthesis can begin by preparing an 5,6-*O*-alkylidene-protected gluconic acid γ-lactone having a protecting group represented by the formula




wherein R is independently alkyl having from 1 to 8 carbon atoms, aryl, or when taken together, form a cycloalkyl ring having from 3 to 8 carbon atoms. The

remainder of the overall synthesis then proceeds as described in accordance with the invention.

**[0030]** As used herein, "alkyl" means both branched- and straight-chain, saturated aliphatic hydrocarbon groups having 1 to 8 carbon atoms. Alkyl groups include, but are not limited to, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and the like. The cycloalkyl ring has from 3 to 8 carbon atoms, and include cyclobutyl, cyclopentyl, cyclohexyl and the like.

**[0031]** As used herein, "aryl" includes "aryl" and "substituted aryl." Thus "aryl" of this invention means any stable 6- to 14-membered monocyclic, bicyclic or tricyclic ring, containing at least one aromatic carbon ring, for example, phenyl, naphthyl, indanyl, tetrahydronaphthyl (tetralinyl) and the like. The presence of substitution on the aryl group is optional, but when present, the substituents can be halo, alkyl, alkoxy, hydroxyl, amino, cyano, nitro, trifluoromethyl, acylamino or carbamoyl, provided that such substituents do not intefere with the synthesis, e.g., by necessitating use of conditions that are not compatible with a step in the synthesis.

**[0032]** As used herein, "stable compound" or "stable structure" means a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture. As those of skill in the art appreciate, the actual chemical stability of each compound will, however, vary depending on the particular substituents and their positions relative to one another. Methods to measure chemical stability are known to those of skill in the art.

**[0033]** Another aspect of the invention provides a method of converting protected DPD to DPD and is applicable for use with any protected DPD molecule, *i.e.*, whether it is a protected DPD made according to the above synthetic pathway or a different pathway and whether it has a different protecting group (including but not limited to alkylidene protection groups described herein or other 1,2-diol protecting groups). To remove the protecting group, an amount of protected DPD in an aqueous solution under acidic conditions is allowed to hydrolyze. Hydrolysis goes to near completion under these conditions (>95% to > 99% complete depending on the duration, temperature and pH. Hydrolysis of at least about 95% or more is considered near completion. The hydrolysis thus produces a stable solution of DPD under the appropriate acidic conditions. The protected DPD can be suspended in an aqueous solution having an acidic pH to allow the reaction to proceed or the protected DPD can be suspended in an aqueous solution which is subsequently acidified to the

desired pH. The pH for hydrolysis is preferably less than about 3, preferably from about 0.5 to 2.0 and more preferably from about 1.3 to about 1.5.

**[0034]** As with the synthetic pathway, the protected DPD can be a racemic mixture or a stereoisomer, *i.e.*, (S)-4,5-cyclohexylidenedioxy-2,3-pentadione or (R)-4,5-cyclohexylidenedioxy-2,3-pentadione (to yield DPD or *ent*-DPD, respectively). After sufficient hydrolysis has occurred, the method further comprises adjusting the pH to about neutral pH or a desired pH range, typically from about pH 7 to about pH 8.5, using a buffering agent suitable for storing DPD or for evaluating the bioactivity of DPD. Preferred pH ranges for evaluating bioactivity are from about pH 7 to about pH 7.5. For storage, the final pH is any value suitable for storing DPD. Stable DPD solutions can be obtained at acidic pH for DPD concentrations that range up to at least 100 mM and possibly higher, depending on the temperature of the solution. A preferred DPD concentration is 30 mM.

**General Discussion of Synthetic Methods**

**[0035]** The synthesis strategy outlined in Fig. 2 constitutes an improvement of that recently reported by Meijler *et al.* (2004) for many reasons. The present invention employs different tactics that significantly improve the synthetic efficiency and make DPD readily available in multigram quantities. The previous synthesis of DPD made use of a labile methyl orthoester as a protecting group, which could be removed in dilute aqueous solution to produce DPD *in situ* for biological evaluation. The delicacy of that protecting group required that it be introduced at a late stage as a replacement for the acetonide group. However, this protection strategy led to complications with the oxidation to form the α-diketone (analog of **11**; 10% yield). In addition, the acetonide series involves two intermediates of inconveniently high volatility.

**[0036]** With access to DPD prepared enzymatically (Schauder *et al.* 2001), the present invention establishes that DPD is stable at pH 1.5-2 in dilute aqueous solution for extended periods (no significant decrease in bioactivity after 16 h at 20 °C for concentrations of DPD at least of 30 mM and up to 100 mM). While enantio-pure glyceraldehyde equivalents provide a convenient conceptual starting point for the synthesis of DPD and other small molecules, the handling of these molecules can be difficult. Isopropylidene glyceraldehyde can be generated as either enantiomer in a

few steps, but it is quite volatile and dimerizes readily (Grauert *et al.* 1985). These drawbacks have led to the development of other protecting groups for glyceraldehyde. For a general discussion, see: Schmid *et al.* 1992; for discussion and elaborate solutions, see: Michel *et al.* 2003 and Aube *et al.* 1992.

**[0037]** The cyclohexylidene group (Fig. 2A) offers lower volatility and efficient isolation, and can be removed rapidly at pH 1.3-1.5 and 20 °C. Importantly, the cyclohexanone byproduct from deprotection does not inhibit cell growth at concentrations under 1 M. The monocyclohexylidene derivative (**7**) of L-gulonic acid γ-lactone was prepared in 75% yield as a white solid (Vekemans *et al.* 1985). Oxidative cleavage with $KIO_4$ led to aldehyde **8** (76%). Following a literature procedure (Yoshida *et al.* 1992), the Corey-Fuchs protocol gave rise to the dibromoalkene **9**, which was purified (67% yield) prior to treatment with *n*BuLi; the intermediate alkyne anion was quenched with methyl iodide to give alkyne **10** in 64% yield after careful chromatography. Alternative quenching with water gave the alkyne **12** (79%); then methylation proceeded in 98% yield to **10**. The critical oxidation process followed the protocol of Seebach (Zibuck *et al.* 1988) with $RuO_2/NaIO_4$ and produced the α-diketone **11** in 70% yield as a bright yellow crystalline solid. The yield is ca 20% overall from gulonic acid γ-lactone *via* **12**. Compound **11** showed a strong tendency to hydrate at the C-3 carbonyl group to give **13** (Fig. 1B), which complicates the characterization. When **11** is placed in $H_2O$ or $D_2O$ at pH 1.5 and 20 °C at concentrations up to 30 mM, deprotection was >95% complete after 2.0 h with no detectable byproducts ($^1H$ NMR). After adjusting the buffer (0.5 M potassium phosphate, pH 7.3), synthetic DPD was observed to be equal in activity to enzymatically prepared DPD at equal concentration in the *V. harveyi* bioassay. Using this route, *ent*-DPD (**14**) and DPD labeled with $^{13}C$ at C-1 (**15**) were synthesized. *Ent*-DPD (**14**) had only 1% of the activity of DPD (**1**) in the *V. harveyi* bioassay.

**[0038]** Synthetically-produced DPD was stable under acidic conditions; a 30 mM sample of the molecule was monitored for decomposition *via* $^1H$ NMR for 5 h at 20 °C and pH 1.5. Under these conditions, no decomposition products were observed. A further examination of a 100 mM sample stored at 20 °C and pH 1.3 for 16 h showed no loss in activity as monitored by the *V. harveyi* bioassay. The purity of the synthetic DPD was further established by reaction with o-phenylenediamine to produce (S)-1-(3-methylquioxalin-2-yl)-ethane-1,2-diol in >98% purity.

**DPD-Borate Binding Studies**

**[0039]** Hydration of the carbonyl group at C-3 of the cyclic form (**2**) of DPD is important for borate binding in the *V. harveyi* signal, **6**. Initially, it was not known whether hydration and subsequent borate addition were intrinsically favorable or required the presence of the protein receptor. For example, Pei and coworkers proposed that the C-3 carbonyl of DPD (enzymatically prepared) was hydrated in aqueous media (Zhu *et al.* 2003).

**[0040]** We showed that laurencione (Fig. 1B), which is 4-deoxy-DPD, hydrates spontaneously and associates strongly with borate in aqueous media at pH 7.8 (Semmelhack *et al.* 2004). It forms complexes with both 1:1 and 2:1 laurencione:borate stoichiometry. For synthetic DPD, the appearance of five $^{13}C$ NMR signals in the spectrum at pH 1.5 (30 mM DPD, aq) in the region from 90-110 ppm assigned to the hemiacetal and hydrated carbons strongly suggests that the majority of DPD, both open and closed isomers, is hydrated at C3. It is less stable at pH > 8 unless bound to borate. Unlike laurencione, which is lacking the C-4 hydroxyl group and therefore in the hydrated form contains only one site for borate complexation, hydrated DPD (**4**) has two sites for borate complexation. Since both 2,3- and 3,4-DPD borates are possible for each of the two anomers, a complex mixture of borate species is expected.

**[0041]** Borate binding was followed with $^{13}C$-labeled DPD, **15**, by both $^{11}B$ and $^{13}C$ NMR spectroscopy. As shown in Fig. 5A, in $H_2O$ (5% $D_2O$) and in the absence of borate, 15 mM **15** produces three main peaks from the incorporated label, assigned to the hydrated open form **16** (δ 24.9 ppm), and the two hydrated cyclic forms, **17/18** (δ 19.9 or 20.4 ppm). As borate is added, the complexity of the $^{13}C$ NMR spectrum increases, consistent with the hypothesis that a number of 1:1 and 2:1 DPD:borate complexes are present in solution; with 15 mM **15** and 45 mM $B(OH)_3$ (Fig. 5B) the signals for **16-18** are gone, confirming a high affinity of these isomers for boron. When the solution is saturated with boric acid (~0.9 M) and the concentration of **15** is kept at 15 mM, the $^{13}C$ NMR spectrum is dominated by a peak at δ 22.3 ppm to which we assign the 2:1 borate complex, **19** (Fig. 5C). Although **19** appears crowded and possesses two negatively charged groups, precedence for this structural type exists in work showing that small polyhydroxylated molecules can

complex multiple tetrahedral phenyboranate ions in close proximity (Lorand *et al.* 1959).

**[0042]** When the identical titration is followed by $^{11}B$ NMR, no signal was observed initially with 15 mM **1** and no $B(OH)_3$ (Fig. 5D), but as the $B(OH)_3$ concentration was increased to 40 mM while holding the concentration of **1** constant (Fig. 5E), new peaks appeared: two broad peaks at δ 4.7 and 5.8 ppm and a family of peaks at δ 8-11 ppm (excess borate at δ 18.3 ppm not shown). The peak at δ 5.8 ppm is assigned to the natural product, **6** (and its anomer), based on analogy with **6** bound to the *V. harveyi* receptor LuxP (6.1 ppm) (Chen *et al.* 2002), **6** released from the LuxP receptor (δ 5.8 ppm) (Miller *et al.* 2004), **6** (δ 5.8 ppm) observed when **17** is released into borate from its receptor, LsrB, from *S. typhimurium* (Miller *et al.* 2004), and the 1:1 borate complex of laurencione (δ 5.9 ppm) (Semmelhack *et al.* 2004).

**[0043]** The peak at δ 4.7 ppm is at a position typical of 1:1 sugar:borate complexes (Van Duin *et al.* 1985) and is tentatively assigned to the 3,4-borate complex, **21**. The family of peaks from δ 8-11 ppm, have positions consistent with a mixture of 2:1 DPD:borate complexes (e.g, **20**) with borate bound at either the 2,3 or 3,4 position (Semmelhack *et al.* 2004). As the concentration of borate is increased to saturation while keeping the concentration of **1** constant at 15 mM, the intensity of the peaks from δ 8-11 ppm decreases while the peaks at δ 4.7 and 5.8 ppm increase and become almost equal in intensity (Fig. 5F).

**[0044]** These data are consistent with the conversion of 2:1 DPD:borate complexes to 1:1 DPD:borate complexes (e.g., **6**), followed by further conversion to 1:2 DPD:borate complex **19** as the borate concentration is increased. The inherently large number of borated DPD species formed coupled with the difficulties associated with accurately measuring the equilibrium constant for sugar borate binding (Springsteen *et al.* 2002) has not allowed quantitation of the equilibrium constant for the association of DPD with borate.

**[0045]** To explore the binding of DPD with borate under conditions that more closely resemble those in the natural habitat of *V. harveyi* and to correlate this study with previous work using DPD produced *in vivo* and released from the *V. harveyi* receptor protein, LuxP, the $^{11}B$ NMR spectrum of a 100 μM DPD solution in 400 μM $B(OH)_3$ (Dickson *et al.* 1990) at pH 7.5 was recorded. Under these conditions, one peak at δ 5.8 ppm corresponding to 1:1 DPD:borate complexes with borate bound at

the 2,3-positions (**6** and its anomer), was observed significantly above noise level. Based on the ratio of integrals for the $B(OH)_3$ peak and the DPD:borate peak, the concentration of DPD:borate complex was calculated to be ca 10 μM. consistent with ca 10% DPD bound at this dilution. We were unable to observe conclusively the presence of complexes with a 2:1 DPD:borate stoichiometry due to sensitivity limitations of the $^{11}B$ NMR experiment, although a peak with a S/N of 2 was recorded at δ 9.8 ppm.

**Intermediates Compounds**

**[0046]** Yet another aspect of the invention provides compounds useful as intermediates in the synthesis of DPD, new borate isomers as well as the isotopically labeled DPD compounds (including the isomeric forms of the isotopically-labeled DPD). These compounds are those shown as compounds **10, 11, 12, 15, 16, 17, 18, 19, 20** and **21** in Figs. 1B and 2A. Where possible, the invention includes the pharmaceutically-acceptable salts of these compounds.

**[0047]** As used herein, "pharmaceutically acceptable salts" refer to derivatives of the disclosed compounds that are modified by making acid or base salts. Examples include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues and the like. Pharmaceutically acceptable salts include, but are not limited to, hydrohalides, sulfates, methosulfates, methanesulfates, toluenesulfonates, nitrates, phosphates, maleates, acetates, lactates and the like.

**[0048]** Pharmaceutically-acceptable salts of the compounds of the invention can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric or greater amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. The salts of the invention can also be prepared by ion exchange, for example. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, the disclosure of which is hereby incorporated by reference in its entirety.

**[0049]** It will be appreciated by those skilled in the art that various omissions, additions and modifications may be made to the invention described above without departing from the scope of the invention, and all such modifications and changes are

intended to fall within the scope of the invention, as defined by the appended claims. All references, patents, patent applications or other documents cited are herein incorporated by reference in their entirety.

## EXAMPLE 1

### General Procedures

**[0050]** A. General. Unless otherwise noted, all reactions were performed under an inert atmosphere with dry reagents, solvents, and flame dried glassware. Reagents were purchased from Acros, Aldrich, or Fluka and used without further purification. With the exception of $CH_3I$, which was distilled before use. Silica flash chromatography was performed using silica gel 60, 230x540 mesh (SORBENT Technologies). Analytical thin layer chromatography was performed using Silica G TLC plate, w/UV254, 200 μm (SORBENT Technologies). The $^1H$ NMR spectra were recorded using a Varian Mercury (300 MHz), Varian Unity (400 MHz), or Varian Unity/INOVA (500 MHz) spectrometer, and $^{13}C$ NMR spectra were recorded using a Varian Unity/INOVA (125.7 MHz) spectrometer. Chemical shifts for $^1H$ and $^{13}C$ NMR spectraare reported in parts per million (ppm) on the δ scale from an internal standard, except for $^{13}C$ NMR recorded in $D_2O$ which were referenced to an external standard. The $^{11}B$ NMR spectra were collected at 20 °C using a Varian Unity/INOVA spectrometer at 160.5 MHz equipped with a 5-mm tunable X/$^1H$ probe (Nalorac), and were referenced indirectly to $BF_3 \bullet Et_2O$. A collection of 3072 scans was averaged for each $^{11}B$ spectrum with a 0.25-s recycle time using an approximately 30° flip-angle pulse. Specific rotations were measured on a PerkinElmer Model 341 Polarimeter using an average of 20 readings for each measurement. High resolution mass spectra were recorded using a Kratos MS 50 RFA.

**[0051]** In the reactions below that indicate preparation of both the L- and D-forms of the compounds, stereoselective synthesis is obtained using the appropriate enantiomer as the starting material or using the stereospecific reaction product from the previous step in the synthesis pathway. For example, in Example 2, starting with L-gulonic acid-γ-lactone yields the final product in its L-form and starting with D-gulonic acid-γ-lactone yields the final product in its D-form.

**[0052]** B. Nomenclature. As shown in Fig. 1A, DPD spontaneously cyclizes to form two epimeric furanoses, (2*S*,4*S*)- and (2*R*,4*S*)- 2,4-dihydroxy-2-

methyldihydrofuran-3-one (*S*- and *R*-DHMF, respectively; also **2** and **3**, respectively). Hydration of *S*- and *R*-DHMF gives rise to (2*S*,4*S*)- and (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*- and *R*-THMF, respectively; also **4** and **5**, respectively).

**[0053]** The IUPAC carbohydrate nomenclature for the structures in Fig. 1A is as follows: DPD, **1**, L-*glycero*-1-dehydro-penta-2,3-diulose; *S*-DHMF, **2**, α-L-*glycero*-1-dehydro-penta-2,3-diulo-2,5-furanose; *S*-THMF, **4**, α-L-*glycero*-1-dehydro-3-hydro-penta-2,3-diulo-2,5-furanose; *S*-THMF-borate, **6**, α-L-*glycero*-1-dehydro-3-hydro-penta-2,3-diulo-2,5-furanosyl-2,3-cyclic borate; *R*-DHMF, **3**, β-L-*glycero*-1-dehydro-penta-2,3-diulo-2,5-furanose; and *R*-THMF, **5**, β-L-*glycero*-1-dehydro-3-hydro-penta-2,3-diulo-2,5-furanose.

## EXAMPLE 2

### Preparation of 5,6-*O*-Cyclohexylidene-L or D-gulono-1,4-lactone, 7 or *ent*-7

**[0054]** To a slurry of gulonic acid-γ-lactone (20.02 g, 112.4 mmol) and *p*-toluene sulfonic acid monohydrate (203 mg, 1.1 mmol) in DMF (100 mL) was added 1,1-dimethoxy cyclohexanone (28.1 g, 29.6 mL, 194.9 mmol) in one portion. The resulting mixture was stirred until the reaction was homogeneous (36-48 h). $Et_3N$ (0.8 mL) was added and stirring was continued for 1 h. The reaction was then concentrated at 60° C *in vacuo* (0.5 mmHg). Addition of PhMe (250 mL) to the warm suspension followed by cooling at –20 °C for 24 h yielded the product as a white precipitate. The precipitate was collected *via* filtration, washed with PhMe, and dried *in vacuo* (0.5 mmHg) for 24 h at room temperature to give **7** (21.65 g, 75% yield). The resulting material contained ~5% starting material and ~1% solvent and was used in subsequent reactions without further purification. $^1$H NMR (300 MHz, DMSO-*d*): δ 5.59 (d, *J* = 7.5 Hz, 1H), 5.46 (d, *J* = 4.2 Hz , 1H), 4.42 (dd, *J* = 4.6, 7.5 Hz 1H), 4.22 (m, 3H), 4.13 (m, 1H), 3.76 (m, 1H), 1.54 (m, 8H), 1.35 (m, 2H). [Vekemans *et al.* 1985.]

## EXAMPLE 3

### Preparation of 2,3-*O*-Cyclohexylidene-L or D-glyceraldehyde, 8 or *ent*-8

**[0055]** To a slurry of $KIO_4$ (10.093 g, 43.90 mmol) and $KHCO_3$ (9.053 g, 90.50 mmol) in $H_2O$ (28 mL) was added **7** (5.050 g, 19.60 mmol). $CH_2Cl_2$, (50 mL) was added, and the resulting two-phase mixture was stirred vigorously for 18 h in air.

At this time, the mixture was diluted with $CH_2Cl_2$ (50 mL), and aqueous NaCl (~0.8 g/mL $H_2O$) was added. Stirring was continued until the aqueous layer gelled. The reaction was filtered, and the residue was washed with $CH_2Cl_2$ (2 x 50 mL). The eluant was collected and washed with $H_2O$ (50 mL). The aqueous fractions were combined and extracted with $CH_2Cl_2$ (2 x 25 mL). The combined organic fractions were dried with $MgSO_4$ and concentrated *in vacuo* giving **8** (2.525 g) in 76% yield and >95% purity. The aldehyde was used without further purification. Due to ABX second order effects, the coupling constants are only reported as 1D approximations. $^1$H NMR (300 MHz, $CDCl_3$): δ 9.73 (d, $J$ = 1.9 Hz, 1H), 4.39 (ddd, $J$ = 1.9, 4.7, 7.3 Hz, 1H), 4.18 (dd, $J$ = 7.3, 8.8 Hz, 1H), 4.10 (dd, $J$ = 4.7, 8.8 Hz, 1H), 1.65 (m, 8H), 1.44 (m, 2H). [Previously characterized by Grauert *et al.* 1985.]

## EXAMPLE 4

### Preparation of (R or S)-1,1-Dibromo-3,4-cyclohexylidenedioxybut-1-ene, 9 or *ent*-9

**[0056]** Triphenyl phosphine (9.181 g, 35.00 mmol) was dissolved in $CH_2Cl_2$ (85 mL) and cooled to 0° C. To the cooled solution was added $CBr_4$ (5.851 g, 17.60 mmol) all at once, and the mixture was stirred for 1 h. At this time a solution of **8** (2.525 g, 14.90 mmol) in 20 mL $CH_2Cl_2$ was added in one portion, and the mixture was stirred for 1 h. The solution was concentrated *in vacuo*, and the residual solid was purified *via* column chromatography ($SiO_2$ eluted with 1:9 EtOAc:hexanes) to give **9** (3.268 g, 67% yield). $R_f$ = 0.83 (1:1, EtOAc:hexanes). $^1$H NMR (300 MHz, $CDCl_3$): δ 6.54 (d, $J$ = 7.7 Hz, 1H), 4.74 (ddd, $J$ = 6.3, 6.5, 7.7 Hz, 1H), 4.19 (dd, $J$ = 6.3, 8.4 Hz, 1H), 3.69 (dd, $J$ = 6.5, 8.4 Hz, 1H), 1.63 (m, 8H), 1.42 (m, 2H). [Previously characterized by Yoshida *et al.* 1992.]

## EXAMPLE 5

### Preparation of (R or S)-1,2-Cyclohexylidenedioxypent-3-yne, 10 or *ent*-10

**[0057]** Dibromoalkene, **9** (1.019 gm 3.10 mmol) was dissolved in THF (20 mL) and subsequently cooled to -78 °C. To this solution was added a solution of nBuLi (2.5 M in hexanes, 3.0 mL, 7.5 mmol) over 15 min *via* an addition funnel. The reaction was allowed to proceed at -78 °C for an additional 30 min. At this time, the solution was warmed to 23 °C and stirred for 1 h. Then, $CH_3I$ (580 μL, 1.32 g, 9.30

mmol ) was added, and stirring was continued for 3 h. The mixture was cooled to 0 °C, quenched with $H_2O$ (100 μL), dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Purification *via* chromatography ($SiO_2$ eluted with 0.5:9.5 $Et_2O$:hexanes) gave **10** (358 mg) in 64% yield. $R_f$ = 0.64 (0.5:9.5, $Et_2O$:hexanes). IR (NaCl) $\nu_{max}$ ($cm^{-1}$): 2936 (s, C-H), 2862 (s, C-H), 2242 (w, C≡C), 1163 (m, C-O), 1105 (s, C-O). **10** $[\alpha]_D^{20}$ –39.2° (*c* 0.767, $CHCl_3$). $^1H$ NMR (500 MHz, $CDCl_3$): δ 4.67 (ddd, *J* = 7.0, 6.1, 2.1 Hz, 1H), 4.10 (dd, *J* = 7.9, 6.1 Hz, 1H), 3.81 (dd, *J* = 7.0, 7.9, 1H), 1.84 (d, *J* = 2.1 Hz, 3H), 1.77 (m, 2H), 1.61 (m, 6H), 1.39 (m, 2H). $^{13}C$ NMR (125.7 MHz, $CDCl_3$): δ 110.7, 82.5, 76.5, 69.9, 65.7, 36.0, 35.6, 25.2, 24.1, 25.2, 24.1, 24.0, 3.7. HRMS (m/z): $M^+$ calcd. For $C_{11}H_{16}O_2$, 180.115; found, 179.635.

**[0058]** The same procedure produced ***Ent*-10:** $[\alpha]_D^{20}$ +39.4° (*c* 0.796, $CHCl_3$).

## EXAMPLE 6

### Preparation of (R)-1,2-Cyclohexylidenedioxybut-3-yne, 12

**[0059]** Vinyldibromide, **9** (1.209 g, 3.70 mmol) was dissolved in THF (20 mL) and subsequently cooled to -78 °C. To this solution was added a solution of nBuLi (2.5 mL in hexanes, 3.6 mL, 9.0 mmol) over 15 min *via* an addition funnel. The reaction was allowed to proceed at -78 °C for 30 min. At this time, the solution was warmed to 23 °C and stirred for 1 h. The mixture was then cooled to 0 °C, quenched with $H_2O$ (100 μL), dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Purification *via* chromatography ($SiO_2$ eluted with 0.5:9.5 $Et_2O$:hexanes) gave **12** (485 mg, 79% yield). $R_f$ = 0.65 (0.5:9.5, $Et_2O$:hexanes). $^1H$ NMR (500 MHz, $CDCl_3$): δ 4.71 (ddd, *J* = 6.5, 6.3, 2.0 Hz, 1H), 4.17 (dd, *J* = 8.0, 6.5 Hz, 1H), 3.95 (dd, *J* = 8.0, 6.3 Hz, 1H), 2.49 (d, *J* = 2.0 Hz, 1H), 1.76 (m, 2H), 1.63 (m, 6H), 1.42 (m, 2H). [Previously characterized by Yoshida *et al.* 1992.]

## EXAMPLE 7

### Preparation of (R)-1,2-Cyclohexylidenedioxypent-3-yne-($^{13}C_5$), 10-($^{13}C_5$)

**[0060]** To a solution of **12** (175 mg, 1.10 mmol) in THF (1.6 mL) at -78 °C was added a solution of nBuLi (2.5 M in hexane, 550 μL, 1.40 mmol) in one portion, and the mixture was allowed to stir for 30 min. At this time, $^{13}CH_3I$ (308 mg, 135 μL, 2.20 mmol) was added, and the mixture was allowed to warm to 23 °C and be stirred for 4 h. The reaction was then cooled to 0 °C and quenched with $H_2O$ (20 μL).

Drying with $MgSO_4$, followed by concentration *in vacuo*, and filtration through silica using 1:2 $Et_2O$:hexanes as the eluant afforded pure **10-($^{13}C_5$)** (186 mg) in 98% yield. $^1H$ NMR (500 MHz, $CDCl_3$): δ 4.67 (ddd, $J$ = 7.0, 6.1, 2.1 Hz, 1H), 4.10 (dd, $J$ = 7.9, 6.1 Hz, 1H), 3.81 (dd, $J$ = 7.0, 7.9, 1H), 1.84 (dd, $J$ = 131.5, 2.1 Hz, 3H), 1.77 (m, 2H), 1.61 (m, 6H), 1.39 (m, 2H). $^{13}C$ NMR (125.7 MHz, $CDCl_3$): δ 110.7, 82.5 (d, $^1J_{(C1)(C2)}$ = 67.8 Hz), 76.5 (d, $^2J_{(C1)(C3)}$ = 11.2 Hz), 69.9, 65.7, 36.0, 35.6, 25.2, 24.1, 25.2, 24.1, 24.0, 3.7.

## EXAMPLE 8

### Preparation of (S or R)-4,5-Cyclohexylidenedioxy-2,3-pentadione, 11 or *ent*-11

**[0061]** To a solution of **10** (330 mg, 1.8 mmol) in $CCl_4$ (4 mL) and MeCN (4 mL) was added $NaIO_4$ (867 mg, 4.1 mmol) in $H_2O$ (6 mL). The mixture was vigorously stirred, and $RuO_2 \bullet H_2O$ (6.0 mg, 4.5 x $10^{-2}$ mmol) was added. The mixture was vigorously stirred for 15 min in air. At this time, it was filtered through silica using $CH_2Cl_2$ as the eluant. The eluant was dried with $MgSO_4$ and concentrated *in vacuo* to give a bright yellow oil. Purification *via* flash chromatography using $SiO_2$ eluted with 1:9 EtOAc:hexanes afforded pure **11** (271 mg) in 70% yield with varying amounts of C3 hydration, i.e. **13** (~10-30%). IR (NaCl) $\nu_{max}$ (mixture of **11** and **13**) ($cm^{-1}$): 3447 (w, br, OH), 2937 (s, CH), 2863 (m, CH), 1794 (w, C=O, hydrated diketone), 1733 (s, C=O, diketone), 1714 (s, C=O, diketone), 1162 (m, C-O), 1145 (m, C-O), 1093 (s, C-O). Azeotropic drying of the oil with $CHCl_3$ produced a yellow solid that contained >95% **11b**. $R_f$ = 0.1-0.3 (1:9 EtOAc: hexanes). $[\alpha]_D^{20}$ -11.8° (*c* 0.900, $CHCl_3$). HRMS (m/z): $M^+$ calcd. For $C_{11}H_{16}O_4$, 212.105; found, 212.645.

**[0062]** In $CDCl_3$, **11** was characterized with <5% **13**. $^1H$ NMR (500 MHz, $CDCl_3$) (**11**): δ 5.14 (dd, $J$ = 5.2, 7.9 Hz, 1H), 4.35 (dd, $J$ = 7.9, 8.9 Hz, 1H), 4.00 (dd, $J$ = 5.2, 8.9 Hz, 1H), 2.40 (s, 3H), 1.64 (m, 8H), 1.42 (m, 2H). $^{13}C$ NMR (125.7 MHz, $CDCl_3$) (**11**): δ 198.2, 194.9, 111.9, 76.5, 65.5, 35.4, 34.7, 25.0, 24.5, 23.82, 23.77.

**[0063]** In aqueous ($D_2O$) solution, **13** formed in >95%, as evidenced by the presence of a new peak at δ 95.7 ppm in the $^{13}C$ NMR spectrum, and the presence of only one peak in the carbonyl region (δ 210.2 ppm). Further evidence for the position of hydration is provided as part of the analytical data for **11/13-($^{13}C_1$)**. $^1H$ NMR (500 MHz, $D_2O$, **13**): δ 4.43 (dd, $J$ = 5.3, 7.2 Hz, 1H), 4.14 (dd, $J$ = 7.2, 9.0 Hz, 1H), 4.05 (dd, $J$ = 5.3, 9.0 Hz, 1H), 2.36 (s, 3H), 1.67-1.30 (m, 10H). $^{13}C$ NMR (125.7 MHz,

$D_2O$, **13**): δ 210.2, 112.1, 95.7, 77.1, 63.8, 34.9, 33.2, 25.1 (splitting due to D exchange was noted), 24.5, 23.6, 23.3. Due to the long exposure to $D_2O$ necessary to obtain the $^{13}C$ NMR spectrum, partial deprotection of the molecule occurred. The weak additional peaks in the $^{13}C$ NMR spectrum (below) of **13** in $D_2O$ at δ 42.0 and 27.0 ppm are due to small amounts of cyclohexanone.

**[0064]** If **11** is dissolved in $D_2O$ and then extracted into $CDCl_3$, >95% **13** could be initially characterized in organic solvent as evidenced by $^1H$ NMR. However, **13** converts to **11** with a $t_{1/2}$ of 13 h at 20 °C. $^1H$ NMR (500 MHz, $CDCl_3$, **13**): δ 4.26 (dd, $J$ = 5.5, 6.7 Hz, 1H), 4.129 (d, $J$ = 5.5 Hz, 1H), 4.127 (d, $J$ = 6.7 Hz, 1H), 2.42 (s, 3H), 1.71-1.56 (m, 8H), 1.41 (m, 2H). $^{13}C$ NMR (125.7 MHz, $CDCl_3$, **13**): (reporting only peaks corresponding to **13**): δ 206.3, 111.2, 94.6, 76.6, 64.6, 35.8, 25.0, 24.5, 24.1, 23.9, 23.7. Due to the length of observation necessary to fully develop the $^{13}C$ spectrum of **13**, considerable dehydration was observed along with deprotection. Therefore, the $^{13}C$ NMR spectrum in $CDCl_3$ of **13** (below) also contains peaks attributed to **11** and cyclohexanone.

**[0065]** ***Ent*-11** $[\alpha]_D^{20}$ +11.5° (*c* 0.733, $CHCl_3$).

## EXAMPLE 9

### Preparation of (S)-4,5-Cyclohexylidenedioxy-2,3-pentadione-($^{13}C_1$), 11-($^{13}C_1$)

**[0066]** Protected DPD, **11**, $^{13}C$ labeled at the C1 position (42 mg, 44% yield) was obtained from **10**-($^{13}C_1$) (81 mg, 0.4 mmol) using the procedure for **11**. $^1H$ NMR (500 MHz, $CDCl_3$): δ 5.14 (dd, $J$ = 5.2, 7.9 Hz, 1H), 4.35 (dd, $J$ = 7.9, 8.9 Hz, 1H), 4.00 (dd, $J$ = 5.2, 8.9 Hz, 1H), 2.40 (d, $J$ = 129.4 Hz, 3H), 1.64 (m, 8H), 1.42 (m, 2H). $^{13}C$ NMR (125.7 MHz, $CDCl_3$): δ 198.2 (d, $^1J_{(C1)(C2)}$ = 42.6 Hz), 194.9(d, $^2J_{(C1)(C3)}$ = 12.4), 111.9, 76.5, 65.5, 35.4, 34.7, 25.0, 24.5, 23.82, 23.77. $^1H$ NMR (500 MHz, $D_2O$): δ 4.43 (dd, $J$ = 5.3, 7.2 Hz, 1H), 4.14 (dd, $J$ = 7.2, 9.0 Hz, 1H), 4.05 (dd, $J$ = 5.3, 9.0 Hz, 1H), 2.36 (d, $J$ = 128.4 Hz, 3H), 1.67-1.30 (m, 10H). $^{13}C$ NMR (125.7 MHz, 0.5:9.5 $D_2O$:$H_2O$): δ 210.2 (d, $^1J_{(C1)(C2)}$ = 41.2), 112.1, 95.7 (d, $^2J_{(C1)(C3)}$ = 11.4), 77.1, 63.8, 34.9, 33.2, 25.1, 24.5, 23.6, 23.3.

**[0067]** The position of hydration in the molecule in aqueous media was confirmed by $^{13}C$ NMR analysis. The peak at δ 95.7 ppm in the $^{13}C$ NMR spectrum for **11b**-($^{13}C_1$) in 5% $D_2O$ in $H_2O$ displayed two bond C-C coupling (11.4 Hz) with the $^{13}C$ label at C1. The chemical shift is also in the range noted for hydrated

carbonyl groups. Taken together, these data are consistent with hydration at C3. Two new peaks at δ 24.6 and 22.3 ppm were also observed in the $^{13}C$ spectrum of **11**-($^{13}C_1$) in 5% $D_2O$ (spectrometer lock signal) in $H_2O$, but not in the $^{13}C$ spectrum of **11** in $D_2O$. These peaks are assigned to the methyl carbon for small amounts of unhydrated and C2 hydrated material that are only detectable with $^{13}C$ incorporation. The two new peaks also show splitting due to D exchange if the $^{13}C$ spectrum is recorded in 100% $D_2O$.

## EXAMPLE 10

### Procedure for initial characterization of 4,5-dihydroxy-2,3-pentadione, 1

**[0068]** To a suspension of **11** (14 mg, 6.6 mmol) in $D_2O$ (3.2 mL) was added conc. $H_2SO_4$ (4 μL, ca pH 2). The reaction was allowed to continue for 2.5 h and was monitored by $^1H$ NMR. Deprotection was determined to be ~90% complete after 1.5 h and greater than ~97% complete after 2.5 h. At this time, excess $NaHCO_3$ and excess $B(OH)_3$ were added to generate a solution of excess borate at pH 7.8. This solution was active in the *V. harveyi* autoinducer-bioassay without further purification. Before addition of $NaHCO_3$ and $B(OH)_3$, a 1 mL aliquot of the material showed an $^1H$ NMR spectrum consistent with previously reported data. $^1H$ NMR (500 MHz, $D_2O$, pD = 2.0): δ 4.36 (dd, $J$ = 5.6, 7.0 Hz, 1H), 4.17 (m, 2H), 4.03 (dd, $J$ = 3.2, 5.9 Hz, 1H), 3.96 (dd, $J$ = 3.8, 7.3 Hz, 1H), 3.80 (m, 2H), 3.63 (dd, $J$ = 7.3, 12.0 Hz, 1H), 3.56 (dd, $J$ = 5.6, 9.4 Hz, 1H) [The previously reported peak at 2.35 (s, 3H) corresponding to the methyl protons of the ring open isomer was obscured by cyclohexanone.], 1.42 (s, 3H), 1.40 (s, 3H). [Previously partially characterized by Meijler *et al.* 2004.]

## EXAMPLE 11

### Procedure for $^1H$-NMR characterization of 4,5-dihydroxy-2,3-pentadione, 1

**[0069]** To a suspension of **11** (9.6 mg, 30 mmol) in $D_2O$ (1.5 mL) was added conc. $D_2SO_4$ (1.5 μL, final pD1.5). The reaction was allowed to continue for 5 h and was monitored by $^1H$ NMR. A $^1H$-$^1H$ DQCOSY spectrum was recorded at the 5-h time point to help establish the identity of each $^1H$ signal. These data are consistent with previous assignments (Janda *et al.* ___). $^1H$ NMR (500 MHz, $D_2O$, pD = 1.5, 5 h): δ 4.36 (dd, $J$ = 5.6, 7.0 Hz, 1H), 4.17 (m, 2H), 4.03 (dd, $J$ = 3.2, 5.9 Hz, 1H), 3.96

(dd, $J$ = 3.8, 7.3 Hz, 1H), 3.80 (m, 2H), 3.63 (dd, $J$ = 7.3, 12.0 Hz, 1H), 3.56 (dd, $J$ = 5.6, 9.4 Hz, 1H) , previously reported peak at 2.35 (s, 3H) corresponding to the methyl protons of the ring open isomer obscured by cyclohexanone, 1.42 (s, 3H), 1.40 (s, 3H). The extra signals in the region form 1.35-1.45 ppm and 3.4-4.6 ppm are most likely due to a small amount of **1** that is not hydrated at C3. This hypothesis is supported by the complete disappearance of these peaks upon addition of 1,2-phneylenediamine (see Example 13)

## EXAMPLE 12

### Procedure for $^{13}C$ NMR characterization of 4,5-dihydroxy-2,3-pentadione, 1

**[0070]** To a suspension of **11** (4.5 mg, 30 mmol) in $H_2O$ (0.665 mL) and $D_2O$ (0.035 mL) was added conc. $D_2SO_4$ (1.5 µL, final pD 1.5). The reaction was allowed to continue for 2.5 h. At this time the $^{13}C$ NMR spectrum was recorded. $^{13}C$ NMR (125.7 MHz, 95:5 $H_2O$:$D_2O$, PH 1.5): ring open isomer (C2 carbonyl not observed): δ 24.9, 61.8, 74.3, 97.2; cyclic isomers: δ 19.9, 20.4, 69.4, 71.3, 73.8, 74.6, 99.4, 99.7, 103.9, 104.7. Note, peaks at δ 24.6, 27.3, 41.9, 221.2 correspond to cyclohexanone. The appearance of 4 $^{13}C$ NMR signals that can be attributed to the ring closed isomers in the region from 90-110 ppm (consistent with signals from hemiacetal and hydrated carbons) strongly suggest that the majority of DPD is hydrated at C3 under the above experimental conditions.

## EXAMPLE 13

### (S)-1-(3-methylquinoxalin-2-yl)-ethane-1,2-diol

**[0071]** To a suspension of **11** (9.6 mg, 30 mmol) in $D_2O$ (1.5 mL) was added conc. $D_2SO_4$ (1.5 µL, final pD1.5), and the reaction was allowed to continue for 5 h. At this time, 400 µL of the solution containing **11** was mixed with 100 µL of a solution of 1,2-phenylenediamine dihydrochloride (17.9 mg, 200 mM) in $D_2O$ (0.5 mL). The resulting solution, containing 24 mM DPD and 40 mM phenylenediamine, was allowed to stand at room temperature for 40 min. The conversion of DPD to (S)-1-(3-methylquinoxalin-2-yl)-ethane-1,2-diol was judged to be >97% *via* $^1H$ NMR. Of interest is the absence of $^1H$ NMR signals in the region from 3.4-4.6 ppm that could be attributed to DPD or a large amount of by-product. $^1H$ NMR (500 MHz, $D_2O$, pD

= 1.5, 5 h): δ 8.31 (m, 1H), 8.14 (m, 1H), 5.42 (dd, $J$ = 4.6, 6.4 Hz, 1H), 4.05 (dd, $J$ = 4.6, 12.0 Hz, 1H), 4.00 (dd, $J$ = 6.4, 12.0 Hz, 1H), 3.02, (s 3H).

## EXAMPLE 14

### Production of (S or R)-4,5-Dihydroxy-2,3-pentadione, 1, suitable for storage and biological evaluation

**[0072]** To a suspension of **11** (10 mM) in $H_2O$ (5 mL) was added conc. $H_2SO_4$ (5 μL). The reaction was allowed to continue for 2 h. At this time, the mixture was diluted with 1 M potassium phosphate buffer (5 mL) to adjust the pH to 7.3, yielding 5 mM DPD, **1**, in 0.5 M potassium phosphate buffer. This material could be used without further purification in the *V. harveyi* bioassay, and displayed an activity equal to that of enzymatically prepared DPD (See next Example).

## EXAMPLE 15

### Bioassay Procedure

**[0073]** *V. harveyi* strain MM32 (*luxN*⁻ *luxS*⁻) was grown overnight and subsequently diluted 5,000-fold in AB growth medium. This bioassay was performed as developed by the method of Schauder et. al. (2001). To 90 μL of diluted cells, 10 μL of various compounds were added, and the resulting cultures were further incubated in a 96-well microtiter dish at 30 °C for approximately 6 h. The concentration of crude DPD was calculated by measuring the amount of homocysteine released in an Ellman's test. Light emission was measured using a Wallac model 1450 microbeta Plus scintillation counter.

**[0074]** A. Comparative bioactivity of enzymatically-produced DPD, synthetic DPD and synthetic *ent*-DPD. Each of these three compounds and a blank were assessed in the above bioassay at equal concentrations. The bio assays were conducted with a dilution of 0.53 mM enzymatically-produced DPD, a dilution of 5.0 mM synthetic DPD or a dilution of 5.0 mM ent-DPD. The results are shown in Fig. 3.

**[0075]** B. Effects of low pH on enzymatically-produced DPD bioactivity. Enzymatically produced DPD (0.53 mM stock concentration) was mixed with sulfuric or hydrochloric acid at a ratio of 1 μL $H_2SO_4$ or 5 μL HCl to 250 μL DPD, resulting in solutions with a pH of 1.5 (with $H_2SO_4$) or 2 (with HCl). At times indicated, 1 M Tris pH 8.5 was added to restore the pH to neutral and bioactivity was

determined. Fig. 4A shows the results after exposure to HCl treatment. Similar results were obtained after exposure to $H_2SO_4$ (not shown). Treatment of enzymatically-produced DPD with acid for up to 16 h did not affect the autoinducer activity.

**[0076]** C. Effects of low pH on synthetic DPD bioactivity. Synthetically-produced DPD (100 mM stock concentration) was mixed with $H_2SO_4$ at a ratio of 1 μL $H_2SO_4$ to 250 μL DPD, resulting in solutions with a pH of 1.3. After 16 h, 1 M Tris pH 8.5 was added at a ratio of 5 μL buffer to 20 uL DPD solution to restore the pH to 7.5. This bioassay carried out as before shows that after treatment of synthetic DPD at a concentration of 100 mM and pH 1.3 for up to 16 h did not change the auto inducer activity (Fig. 4B).

## EXAMPLE 16

### $^{13}C$ NMR DPD-borate binding study using DPD-($^{13}C_1$), 15

**[0077]** To a suspension of **11**-($^{13}C_1$) (5.2 mg , 0.025 mmol) in 0.5:9.5 $D_2O$:$H_2O$ (1.64 mL ) was added conc. $H_2SO_4$ (2 μL). The reaction was allowed to continue for 2.0 h, and the $^{13}C$ NMR shifts for the methyl groups of the equilibrium mixture were recorded. Excess $NaHCO_3$ and $B(OH)_3$ (4.6 mg, 0.074 mmol) were added to generate a sat. $NaHCO_3$ and 45 mM $B(OH)_3$ solution (pH 7.8), and the $^{13}C$ NMR spectrum of the resulting mixture was recorded. Excess $B(OH)_3$ was added to the mixture to generate a sat. $B(OH)_3$ solution, and the sample was further analyzed *via* $^{13}C$ NMR spectroscopy. The results are shown in Fig. 5A-C and discussed in the Detailed Description of the Invention.

## EXAMPLE 17

### $^{11}B$ NMR DPD-borate binding study

**[0078]** To a suspension of **11** (8.1 mg, 0.038 mmol) in 0.5:9.5 $D_2O$:$H_2O$ (1.53 mL) was added conc. $H_2SO_4$ (2 μL). The deprotection was allowed to continue for 2 h. At this time, excess $NaHCO_3$ was added to generate a sat. $NaHCO_3$ solution (pH 7.8). The material was divided into aliquots, and 100 mM $B(OH)_3$ in sat $NaHCO_3$ (aq) and sat $NaHCO_3$ (aq) were added in various proportions to generate 15 mM DPD in 5 mM, 15 mM, and 40 mM $B(OH)_3$ solutions with final volumes of 0.5 mL. These solutions were studied by $^{11}B$ NMR. Excess $B(OH)_3$ was added to the 45 mM $B(OH)_3$ sample to generate a saturated $B(OH)_3$ solution, and this sample was

subjected to further $^{11}B$ NMR analysis. The results are shown in Fig. 5D-F and discussed in the Detailed Description of the Invention.

**[0079]** To explore further the ability of DPD to complex borate at more physiologically relevant concentrations, a mixture of 1 0 μL of 40 mM $B(OH)_3$, 10 μL of 10 mM DPD, 100 μL of 1 M potassium phosphate buffer (pH 7), 200 μL of sat. $NaHCO_3$ and 680 μL of $D_2O$ was produced to generate a 100 μM DPD solution in 400 μM $B(OH)_3$, at pH 7.5. This solution was then analyzed by $^{11}B$ NMR to determine binding, as discussed in the Detailed Description of the Invention.

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WE CLAIM:

1. A method to synthesize protected DPD which comprises

a) reacting gluconic acid γ-lactone with dimethoxycyclohexanone for a time and under conditions to produce 5,6-*O*-cyclohexylidene-glucono-1,4-lactone;

b) subjecting said lactone to oxidative cleavage to produce 2,3-*O*-cyclohexylidene-glyceraldehyde;

c) halogenating said glyceraldehyde under conditions to produce 1,1-dihalo-3,4-cyclohexylidenedioxybut-1-ene ("dihaloalkene");

d) purifying said dihaloalkene and further reacting said dihaloalkene under conditions to convert the alkene moiety of said dihaloalkene to an alkyne anion;

e) quenching said anion with either

i) a methylating agent to produce 1,1-dihalo-3,4-cyclohexylidenedioxypent-3-yne, or

ii) water to produce 1,1-dibromo-3,4-cyclohexylidenedioxybut-3-yne, followed by methylation to produce 1,1-dibromo-3,4-cyclohexylidenedioxypent- 3-yne,

and recovering said alkyne;

f) oxidizing said alkyne in non-aqueous solution under conditions to produce 4,5-cyclohexylidenedioxy-2,3-pentadione, which is said protected DPD; and

g) recovering said protected DPD.

2. The method of Claim 1, wherein said gluconic acid γ-lactone is a racemic mixture of the L- and D- stereoisomers of gluconic acid γ-lactone and said protected DPD is a racemic mixture of (S)-4,5-cyclohexylidenedioxy-2,3-pentadione and (R)-4,5-cyclohexylidenedioxy-2,3-pentadione.

3. A method to stereospecifically synthesize protected DPD which comprises

a) reacting the desired stereoisomer of gluconic acid γ-lactone with dimethoxycyclohexanone for a time and under conditions to produce 5,6-*O*-cyclohexylidene-glucono-1,4-lactone;

b) subjecting said lactone to oxidative cleavage to produce 2,3-*O*-cyclohexylidene-glyceraldehyde;

c) halogenating said glyceraldehyde under conditions to produce 1,1-dihalo-

3,4-cyclohexylidenedioxybut-1-ene ("dihaloalkene");

d) purifying said dihaloalkene and further reacting said dihaloalkene under conditions to convert the alkene moiety of said dihaloalkene to an alkyne anion;

e) quenching said anion with either

i) a methylating agent to produce 1,1-dihalo-3,4-cyclohexylidenedioxypent-3-yne, or

ii) water to produce 1,1-dibromo-3,4-cyclohexylidenedioxybut-3-yne, followed by methylation to produce 1,1-dibromo-3,4-cyclohexylidenedioxypent- 3-yne,

and recovering said alkyne;

f) oxidizing said alkyne in non-aqueous solution under conditions to produce 4,5-cyclohexylidenedioxy-2,3-pentadione, which is said protected DPD; and

g) recovering said protected DPD having the desired stereospecificity.

4. The method of claim 1, wherein said gluconic acid γ-lactone is L-gluconic acid γ-lactone and said protected DPD is (S)-4,5-cyclohexylidenedioxy-2,3-pentadione.

5. The method of Claim 1, wherein said gluconic acid γ-lactone is D-gluconic acid γ-lactone and said protected DPD is (R)-4,5-cyclohexylidenedioxy-2,3-pentadione.

6. A method of converting protected DPD to DPD which comprises maintaining an amount of said protected DPD in an aqueous solution under acidic conditions, and allowing hydrolysis to proceed for a time sufficient to remove the protecting group and produce a stable solution of DPD.

7. The method of Claim 6, wherein said protected DPD is suspended in an aqueous solution having an acidic pH.

8. The method of Claim 6, wherein said protected DPD is suspended in an aqueous solution and said solution is subsequently acidified.

9. The method of any one of Claims 6-8, wherein said acidic conditions provide a final pH less than about 3.

10. The method of any one of Claims 6-8, wherein said acidic conditions provide a final pH of about 0.5 to about 2.0.

11. The method of any one of Claims 6-8, wherein said acidic conditions provide a final pH of about 1.3 to about 1.5.

12. The method of Claim 6, wherein said protecting group is a alkylidene group.

13. The method of Claim 12, wherein said protecting group is a cyclohexylidene group.

14. The method of Claim 6, wherein said protected DPD is (S)-4,5-cyclohexylidenedioxy-2,3-pentadione, (R)-4,5-cyclohexylidenedioxy-2,3-pentadione or a racemic mixture thereof.

15. The method of Claim 6, wherein after sufficient hydrolysis has occurred, the method further comprises adjusting said pH to about neutral pH using a buffering agent suitable for storing DPD or for evaluating the bioactivity of DPD.

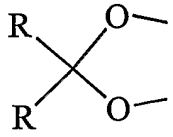
16. The method of Claim 6, wherein after sufficient hydrolysis has occurred, the method further comprises maintaining said pH at a value suitable for storing DPD or for evaluating the bioactivity of DPD.

17. The method of Claim 1 or 3, wherein isotopically labelled compounds are produced by using a methylating agent with an isotope of carbon-12 in step e).

18. The method of Claim 17, wherein the isotope is $^{13}C$ or $^{14}C$.

19. A method to synthesize protected DPD which comprises

a) preparing an 5,6-*O*-alkylidene-protected gluconic acid γ-lactone having a protecting group represented by the formula




wherein R is independently alkyl having from 3 to 8 carbon atoms, aryl, or when taken together, form a cycloalkyl ring having from 4 to 8 carbon atoms;

b) subjecting said lactone to oxidative cleavage to produce 2,3-*O*-alkyideneglyceraldehyde;

c) halogenating said glyceraldehyde under conditions to produce a 1,1-dihalo-3,4-alkylidenedioxybut-1-ene ("dihaloalkene");

d) purifying said dihaloalkene and further reacting said dihaloalkene under conditions to convert the alkene moiety of said dihaloalkene to an alkyne anion;

e) quenching said anion with either

i) a methylating agent to produce 1,1-dihalo-3,4-alkylidenedioxypent-3-yne, or

ii) water to produce 1,1-dibromo-3,4-alkylidenedioxybut-3-yne,

followed by methylation to produce 1,1-dibromo-3,4-alkylidenedioxypent-3-yne,

and recovering said alkyne;

f) oxidizing said alkyne in non-aqueous solution under conditions to produce 4,5-alkylidenedioxy-2,3-pentadione, which is said protected DPD; and

g) recovering said protected DPD.

20. A compound which one of compounds **10, 11, 12, 15, 16, 17, 18, 19, 20** or **21** of Figs. 1B and 2A, and pharmaceutically acceptable salts thereof.

21. The compound of Claim 20 which is compound **10, 11** or **12.**

22. The compound of Claim 21, wherein said compound is isotopically-labeled at a carbon position.

23. The compound of Claim 22, wherein said carbon is at the same position as the C1 position of DPD.

24. The compound of Claim 23, wherein the isotope is $^{13}C$ or $^{14}C$.

25. Isotopically-labeled DPD or *ent*-DPD, said label being at a carbon position.

26. The compound of Claim 25, wherein said carbon is at the C1 position of DPD.

27. The compound of Claim 26, wherein the isotope is $^{13}C$ or $^{14}C$.

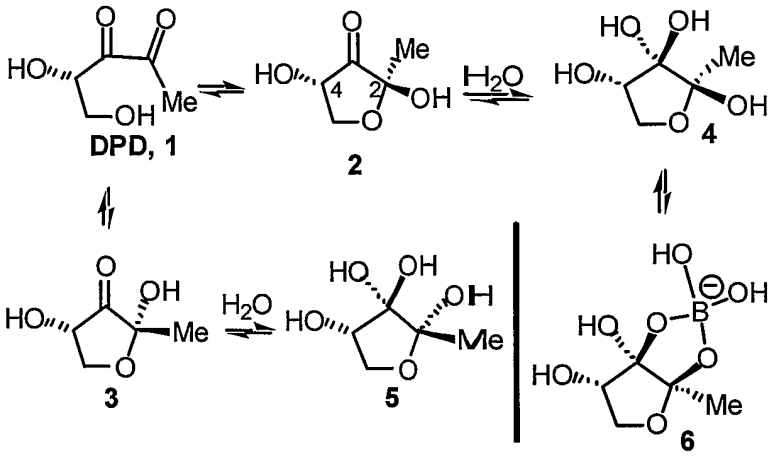



Figure 1A

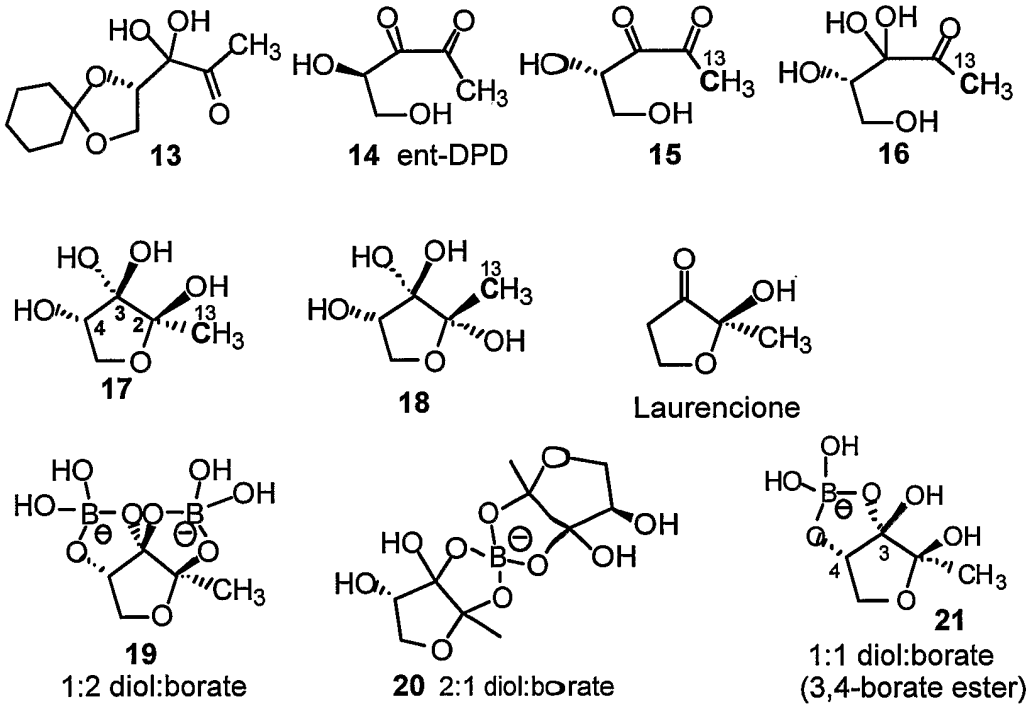



Fig. 1B

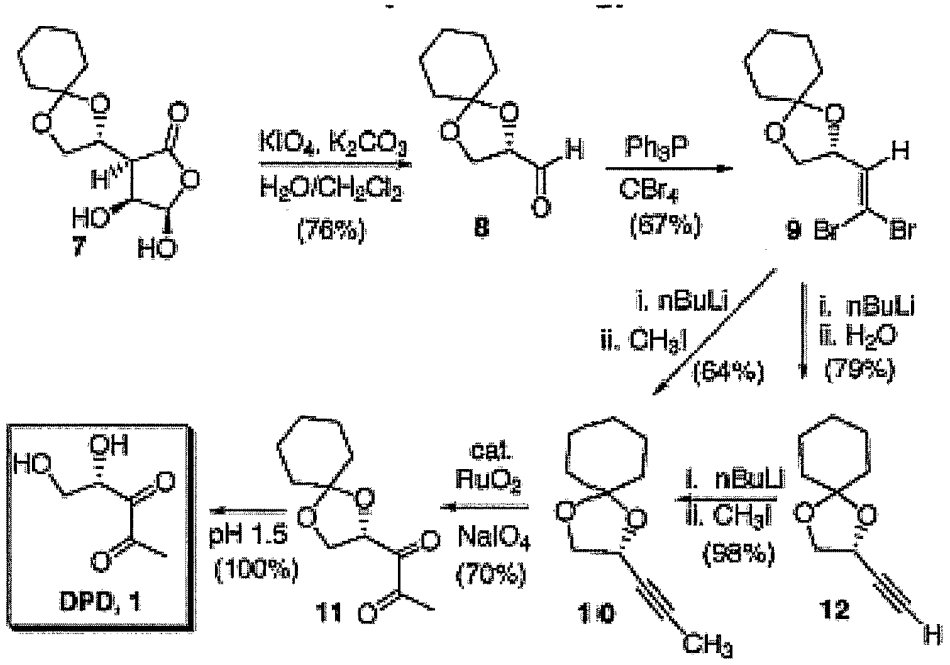



Figure 2A

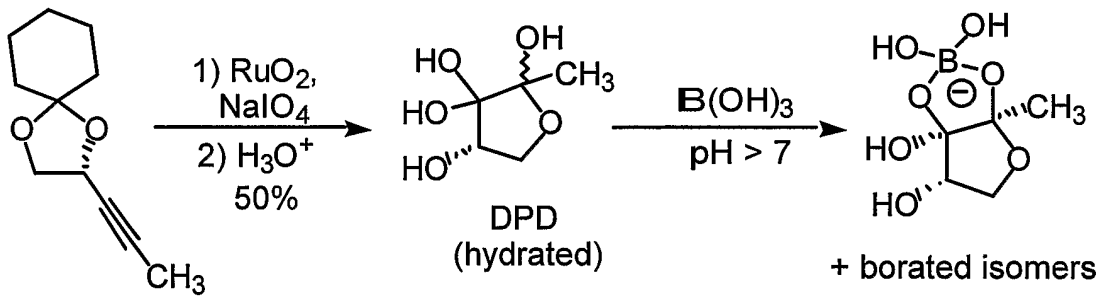



Fig 2B

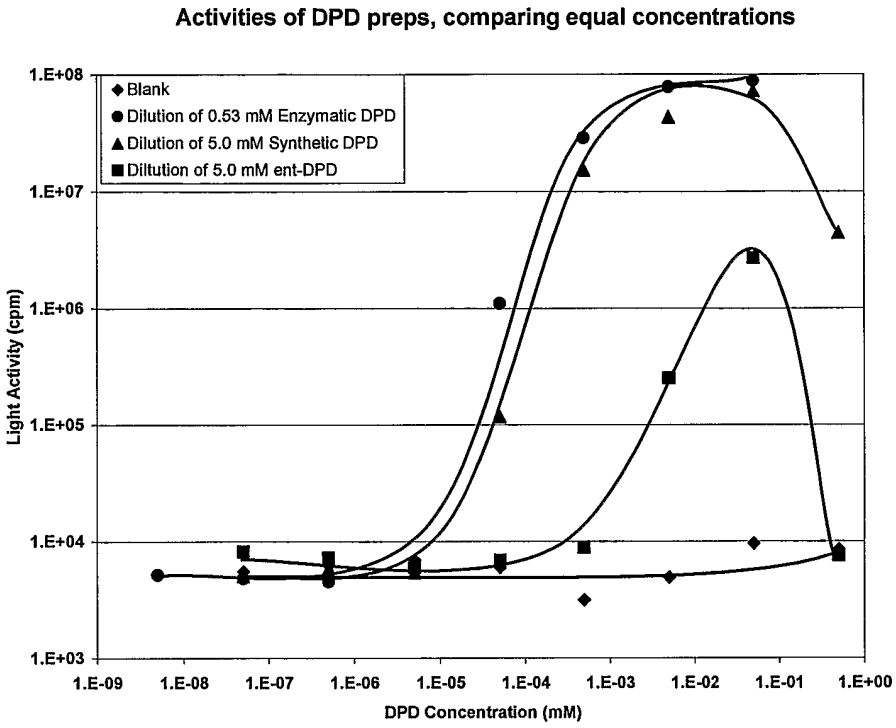

Activities of DPD preps, comparing equal concentrations
Blank
Dilution of 0.53 mM Enzymatic DPD
Dilution of 5.0 mM Synthetic DPD
Diltution of 5.0 mM ent-DPD
Light Activity (cpm)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
1.E-04
1.E-03
1.E-02
1.E-01
1.E+00
DPD Concentration (mM)

Fig. 3

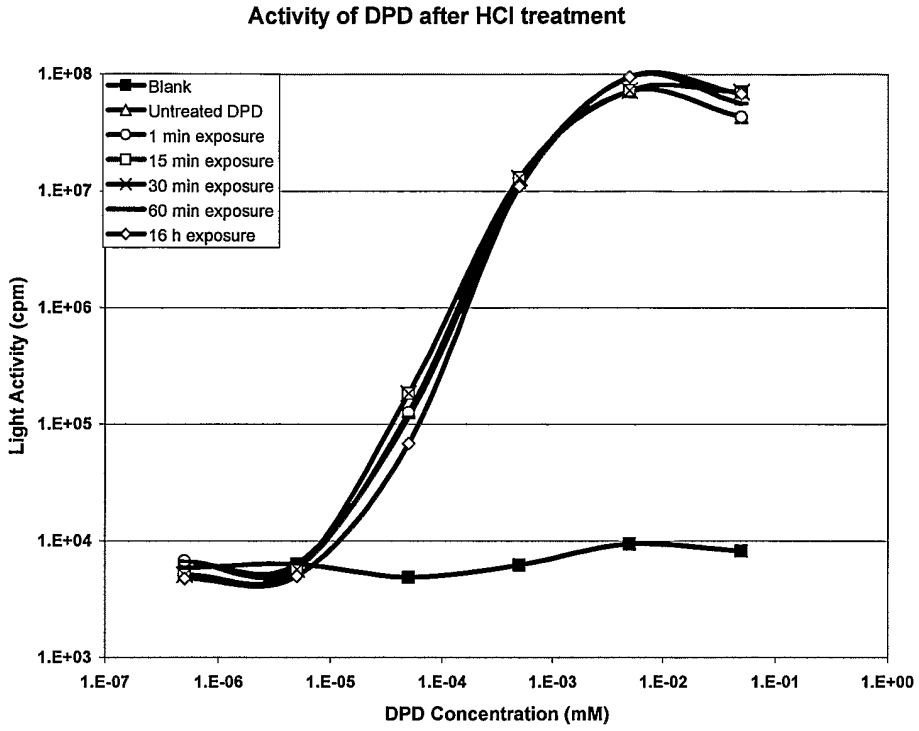

Activity of DPD after HCl treatment
Blank
Untreated DPD
1 min exposure
15 min exposure
30 min exposure
60 min exposure
16 h exposure
Light Activity (cpm)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E-07
1.E-06
1.E-05
1.E-04
1.E-03
1.E-02
1.E-01
1.E+00
DPD Concentration (mM)

Fig. 4A

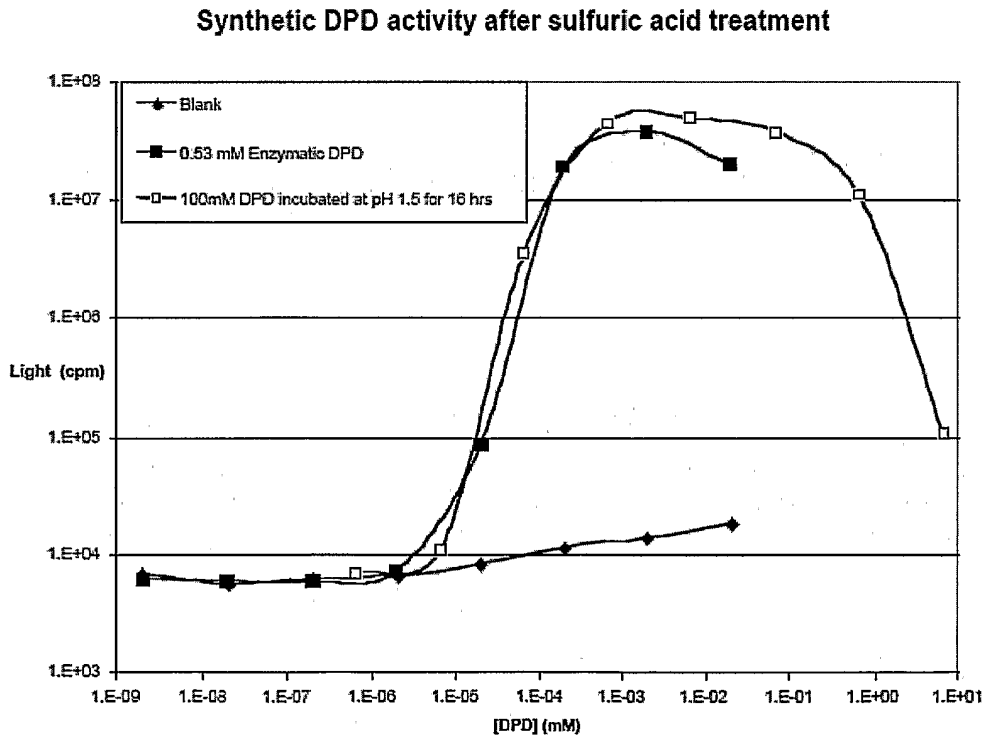

Synthetic DPD activity after sulfuric acid treatment
Blank
0.53 mM Enzymatic DPD
100mM DPD incubated at pH 1.5 for 16 hrs
Light (cpm)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
1.E-04
1.E-03
1.E-02
1.E-01
1.E+00
1.E+01
[DPD] (mM)

Fig. 4B

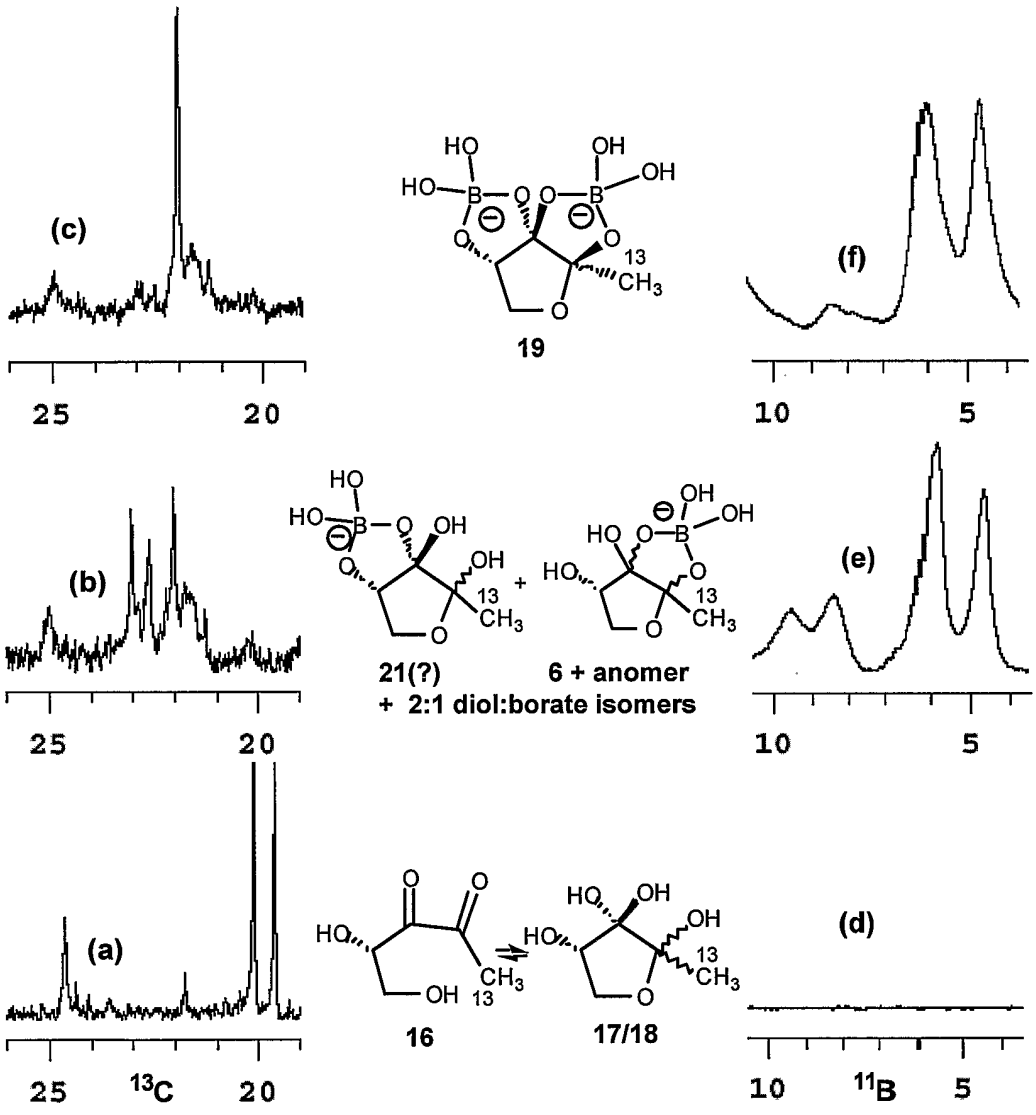

(c)
25
20
19
(f)
10
5
(b)
25
20
21(?)
6 + anomer
+ 2:1 diol:borate isomers
(e)
10
5
(a)
25
13C
20
16
17/18
(d)
10
11B
5

Fig 5

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/33648

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) : C07C 45/00, 49/12; C07D 307/00

US CL : 568/343, 347, 348, 388, 413; 549/430, 476

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)

U.S. : 568/343, 347, 348, 388, 413; 549/430, 476

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)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | ZHU et al. Catalytic Mechanism of S-Ribosylhomocysteinase (LUXS): Stereochemical Course and Kinetic Isotope Effect of Proton Transfer Reactions. Biochemistry. 2004, vol. 43, pages 10166-10172, see entire document. | 20 |
| X<br>---<br>Y | SEMMELHACK et al. Boron Binding with the Quorum Sensing Signal AL-2 and Analogues. Organic Letters. 2004, vol. 6, no. 15, pages 2635-2637, see entire document. | 20<br>---<br>20 |
| X | WU et al. Synthesis and Biological Evaluation of 1 alpha-hydroxy-25 (R and S)-25, 26-Epoxy-23-yne Vitamin D3 and of 1 alpha, 25 (R and S), 26-Trihydroxy-23-yne Vitamin D3. Bioorganic & Medicinal Chemistry Letters. 1997, vol. 7 no. 7, pages 929-932, see entire document. | 20,21 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"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

"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

"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

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Date of the actual completion of the international search

13 January 2006 (13.01.2006)

Date of mailing of the international search report

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Continuation of B. FIELDS SEARCHED Item 3:
REIGISTRY, HCAPLUS, HCAOLD, BIOSIS, MEDLINE
search tems: dihydroxypentanedione, protected, unprotected, radio-labeled, isotope, gluconic acid gamma lactone, glyceraldehyde, halogenate, chlorinate, fluorinate, brominate, alkyne, quenching, methylating agent, water, steam.