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Research Article

Synthesis of ^{3}H- and $^{2}H_4$-labelled versions of the hypoxia-activated pre-prodrug 2-((2-bromoethyl)-2,4-dinitro-6-(((2-(phosphonooxy)ethyl)amino)carbonyl)anilino)ethyl methanesulfonate (PR-104)

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Abstract: ^{3}H- and $^{2}H_4$-versions of the hypoxia-activated pre-prodrug PR-104 [2-[(2-bromoethyl)-2,4-dinitro-6-[[[2-(phosphonooxy)ethyl]amino]carbonyl]anilino]ethyl methanesulfonate], labelled in the ethylcarboxamide side chain, have been prepared, respectively, by [^{3}H]$NaBH_4$ reduction of a precursor late stage aldehyde, and by late stage incorporation of deuterium with 2-amino[1,1,2,2-$^{2}H_4$]ethanol. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: dinitrobenzamide mustard; hypoxia-activated prodrug; PR-104; tritium; deuterium

Introduction

The asymmetric mustard dinitrobenzamide phosphate PR-104 (**1**)[1,2] is a soluble pre-prodrug designed to be converted rapidly into the prodrug **2** by serum phosphatases (Scheme 1). This alcohol then diffuses efficiently to, and is activated selectively in, hypoxic regions of solid tumors[3] to reactive reduced species (e.g. **3** and **4**) that are cytotoxic DNA cross-linking agents.[4] PR-104 shows excellent *in vivo* activity against the subfraction of hypoxic cells in human tumor xenografts,[1,2] and has recently begun Phase I clinical trials.

As part of the preclinical development of **1**, a tritium-labelled version was required for drug disposition and metabolic studies. As previous work[4] had shown that oxidative loss of one or both arms of the mustard was a minor pathway in the metabolism of compounds related to **1**, it was decided to place the label in the carboxamide side chain. This had the additional benefit of being able to start with prodrug **2** as the late stage intermediate. A stably labelled version of **1** was also required, to use as an internal standard for the mass spectrometric analysis of preclinical and clinical plasma samples. The presence of natural isotopes of the bromine in **1** necessitated a standard that differed by at least three mass units, and a synthesis that exploited the late stage introduction of commercially available tetra-deutero 2-aminoethanol was devised.

Discussion

The synthesis of tritium-labelled **1** (**1-T**) is shown in Scheme 2. A number of oxidizing agents were evaluated for the conversion of alcohol **2** to aldehyde **5**, the best being Dess-Martin periodinane and the eventually employed pyridinium chlorochromate (PCC). Reduction of **5**, used in excess) with [^{3}H]$NaBH_4$ (60 Ci/mmol, diluted two-fold with unlabelled $NaBH_4$) in ethanol/THF at 20°C for 20 min gave the labelled alcohol **6** in good yield, with a measured specific activity of 6.3 Ci/mmol. Compound **6** (diluted 10-fold with unlabelled **2**) was phosphorylated as previously reported[1] for **2**, with di-tert-butyl diisopropylphosphoramidite/1*H*-tetrazole followed by oxidation with 70% hydrogen peroxide, to provide the phosphate ester **7**. This was cleaved with trifluoroacetic acid to give phosphate diacid **1-T** in 65% overall yield from **6**, with a measured specific activity of 0.77 Ci/mmol.

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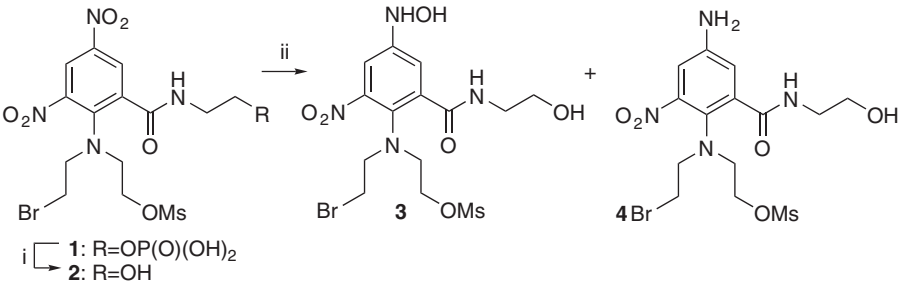
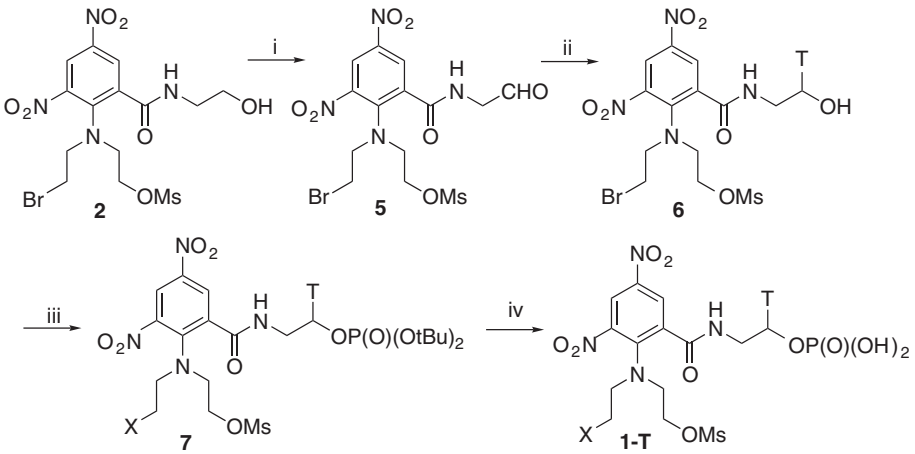


(i) Serum phosphatases; (ii) cellular reductases under hypoxia.

Scheme 1 Mechanism of activation of PR-104.



Reagents: (i)PCC; (ii) [T]$NaBH_4$/EtOH; (iii) i-$Pr_2NP(OtBu)_2$/1*H*-tetrazole, then H_2O_2; (iv) TFA/CH_2Cl_2

Scheme 2 Synthesis of 3H PR-104.

For analysis and quantitation of the preclinical and clinical plasma samples from treatment with **1** by mass spectrometry, an internal standard, differing by at least three mass units, was required. The commercial availability of tetra-deuterated 2-aminoethanol allowed the opportunity of incorporating four D atoms in the carboxamide side chain to give **1-D4**. Rather than repeat the standard synthesis,[1] which involved introduction of the side chain early in the synthesis, an alternative route was devised where the side chain was incorporated as late as possible (after elaboration of the mustard group) (Scheme 3).

In this procedure, the known[5] *tert*-butyl ester **8** was reacted with diethanolamine to give the diol **9**, which was converted to the dimesylate **10** with mesyl chloride in pyridine. This was treated with lithium bromide in DMF, and the resulting bromomesylate **11** was separated from the corresponding dibromo compound and unreacted **10** by column chromatography. The ester **11** was then cleaved with trifluoroacetic acid to give the key acid **12** in 33% overall yield from **8**.

Activation of acid **12** with a number of coupling reagents (DECP, CDI, EDCI) proved unsuccessful. However, conversion of **12** to the corresponding acid chloride (oxalyl chloride, catalytic DMF) followed by reaction with 2-amino[1,1,2,2-$^2H_4$]ethanol in the presence of triethylamine gave the labelled alcohol **13** in good yield. HPLC analysis showed this contained 4.4% of the corresponding chloromesylate, generated by chloride exchange of the bromide. The alcohol **13** was phosphorylated with di-*tert*-butyl diisopropylphosphoramidite/1*H*-tetrazole as previously described[1] for **2**, and the resulting phosphate ester **14** was cleaved with trifluoroacetic acid to give phosphate diacid **1-D4**, in 16% overall yield from **8**. Deuterium incorporation (98%) was confirmed by high resolution FAB mass spectrometry.

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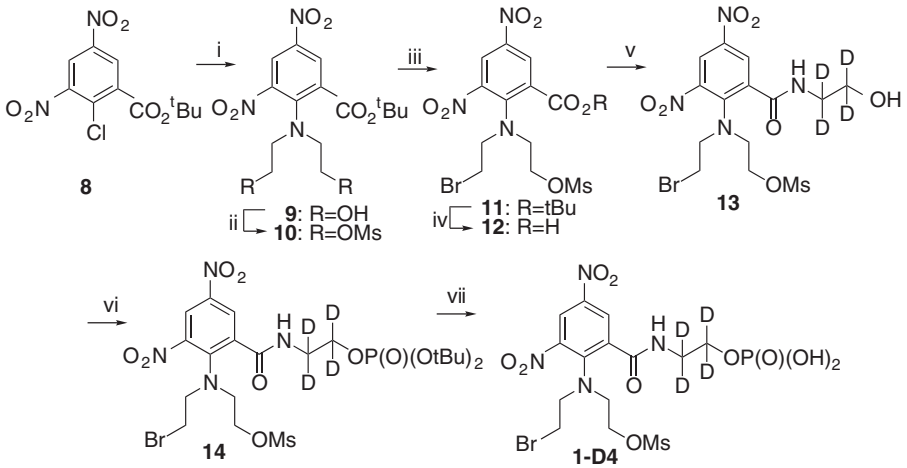

Reagents: (i) $HN(CH_2)CH_2OH)_2$; (ii) MsCl; (iii) LiBr; (iv) TFA; (v) $(COCl)_2$, then $H_2N(CD_2)_2OH$; (vi) i-$Pr_2NP(OtBu)_2$/1*H*-tetrazole, then H_2O_2; (vii) TFA/CH_2Cl_2

Scheme 3 Synthesis of 2H4 PR-104.

Experimental section

Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined using an Electrothermal Model 9200 digital melting point apparatus, and are as read. NMR spectra were measured on a Bruker DRX–400 spectrometer, and referenced to Me_4Si. Mass spectra were recorded on a Varian VG-70SE spectrometer. HPLC was carried out using a Bondclone 10 C18 reverse-phase silica gel column, with a Phillips PU4100 M gradient elution pump and a Phillips PU 4120 diode array detector, and eluting with the appropriate ratios of 95% MeCN/5% water (solvent A) and 0.45 M ammonium formate buffer (solvent B; pH 3.45). The specific activities of **6** and **1-T** were determined by quantitation of ^{3}H-labelled samples by HPLC, and subsequent off-line liquid scintillation counting (Packard 1500 Tri-Carb) of the isolated HPLC peaks.

2-((2-Bromoethyl)-2,4-dinitro-6-(((2-oxoethyl)amino)-carbonyl)anilino)ethyl methanesulfonate (5)

A solution of 2-[(2-bromoethyl)-2-[[(2-hydroxyethyl)amino]carbonyl]-4,6-dinitroanilino]ethyl methanesulfonate[1] (**2**) (600 mg, 1.20 mmol) in dry CH_2Cl_2 (125 ml) was stirred with 3A powdered molecular sieves (1.5 g) for 5 min at room temperature, then treated with powdered pyridinium chlorochromate (777 mg, 3.60 mmol). The mixture was stirred at room temperature for a further 15 min, then treated with water (50 ml) and filtered. The organic phase was washed with water (50 ml), dried and concentrated under reduced pressure. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether (4:1). The middle fractions were combined and rechromatographed, eluting with EtOAc/petroleum ether (6:1) to give **5** (413 mg, 69%) as a yellow gum which was used directly: ^{1}H NMR [$(CD_3)_2SO$] δ 9.61 (s, 1H), 9.15 (t, $J = 5.4$ Hz, 1H), 8.77 (d, $J = 2.8$ Hz, 1H), 8.42 (d, $J = 2.8$ Hz, 1H), 4.28 (t, $J = 5.4$ Hz, 2H), 4.20 (t, $J = 5.5$ Hz, 2H), 3.62–3.54 (m, 2H), 3.54–3.43 (m, 4H), 3.12 (s, 3H).

(^{3}H)2-((2-Bromoethyl)-2-(((2-hydroxyethyl)amino)-carbonyl)-4,6-dinitroanilino)ethyl methanesulfonate (6)

A freshly prepared solution of [^{3}H]$NaBH_4$ (0.84 mg, 0.02 mmol, nominally 1.2 Ci at 60 Ci/mmol) (Amersham Biosciences, UK) and unlabelled $NaBH_4$ (0.76 mg, 0.02 mmol) in EtOH (1.5 ml) was added dropwise to a stirred solution of **5** (99 mg, 0.20 mmol) in THF (5 ml) at 20°C over a period of 5 min. The mixture was stirred at 20°C for a further 15 min, then quenched with 0.1 N aqueous MsOH (1 ml), diluted with water (20 ml) and extracted with EtOAc (2×20 ml). The combined organic layer was washed with water (2×20 ml), dried, and concentrated under reduced pressure. Chromatography on silica gel, eluting with EtOAc/petroleum ether (6:1), gave unreacted **5** (8 mg), then **6** (60 mg, 75%); (6.3 Ci/mmol) as a yellow gum, identical on HPLC with unlabelled **2**: ^{1}H NMR [$(CD_3)_2SO$] δ 8.76–8.70 (m, 2H), 8.35 (d, $J = 2.8$ Hz,

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1H), 4.83–4.75 (m. 1H), 4.28 (t, $J = 5.5$ Hz, 2H), 3.61–3.52 (m, 4H), 3.51–3.42 (m, 4H), 3.39–3.31 (m, 2H), 3.13 (s, 3H).

(^{3}H)2-((2-Bromoethyl)-2-(6-*tert*-butoxy-8,8-dimethyl-6-oxido-5,7-dioxa-2-aza-6-phosphanon-1-anoyl)-4,6-dinitroanilino)ethyl methanesulfonate (7)

A stirred solution of **6** (30 mg, 0.06 mmol; 235 GBq/mmol [6.3 Ci/mmol] and unlabelled **2** (270 mg, 0.54 mmol) in dry DMF (1.5 ml) at 10°C was treated with 1H-tetrazole (67 mg, 0.96 mmol), followed by the dropwise addition of di-tert-butyl diisopropylphosphoramidite (0.26 ml, 0.78 mmol), 95%). The mixture was warmed to room temperature of 2 h, then cooled to 0°C and treated slowly with a solution of 70% aqueous H_2O_2 (0.15 ml) in THF (0.2 ml). The mixture was stirred at 10°C for a further 30 min, then poured into cold water (25 ml) and extracted with EtOAc (2 × 10 ml). The combined organic layers were washed with 5% aqueous $Na_2S_2O_5$ (10 ml), water (4 × 10 ml), dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether, and the eluant was concentrated under reduced pressure below 30°C to a small volume and diluted with hexane to precipitate **7** (303 mg, 73%) as a yellow oil: ^{1}H NMR [$(CD_3)_2SO$] δ 8.93 (t, $J = 5.6$ Hz, 1H), 8.75 (d, $J = 2.8$ Hz, 1H), 8.34 (d, $J = 2.8$ Hz, 1H), 4.28 (t, $J = 5.4$ Hz, 2H), 4.06–3.97 (m, 2H), 3.61–3.43 (m, 8H), 3.13 (s, 3H), 1.43 (s, 18H).

(^{3}H)2-((2-Bromoethyl)-2,4-dinitro-6-(((2-(phosphonooxy)ethyl)-amino)carbonyl)anilino)ethyl methanesufonate (1-T)

A solution of **7** (299 mg, 0.43 mmol) in CH_2Cl_2 (2 ml) was treated with TFA (2 ml) at room temperature for 1 h, then concentrated to near dryness under reduced pressure below 30°C. The residual gum was dissolved in dry MeCN (0.2 ml) and the solution was diluted with dry CH_2Cl_2 (10 ml), filtered, seeded, and refrigerated at 5°C for 16 h. The separated solid was collected, washed with CH_2Cl_2 and dried under high vacuum to give **1-T** (223 mg, 89%; 98.5% pure by HPLC) (0.77 Ci/mmol) as yellow crystals. ^{1}H NMR [$(CD_3)_2SO$] δ 11.0 (br, 2H), 8.97–8.90 (m, 1H), 8.74 (d, $J = 2.8$ Hz, 1H), 8.37 (d, $J = 2.8$ Hz, 1H), 4.29 (t, $J = 5.4$ Hz, 2H), 3.98 (q, $J = 6.2$ Hz, 2H), 3.62–3.43 (m, 8H), 3.13 (s, 3H).

tert-Butyl 2-(bis(2-hydroxyethyl)amino)-3,5-dinitrobenzoate (9)

A solution of *tert*-butyl 2-chloro-3,5-dinitrobenzoate[5] (**8**) (3.27 g, 10.8 mmol) in dioxane (10 ml) at 10°C was treated with diethanolamine (2.84 g, 27 mmol) and vigorously stirred at room temperature for 3 h. Dilution with water provided a solid which was purified by column chromatography on silica gel, eluting with EtOAc/petroleum ether (4:1), followed by recrystallization from EtOAc/iPr_2O to give *tert*-butyl 2-[bis(2-hydroxyethyl)amino]-3,5-dinitrobenzoate (**9**) (3.78 g, 94%) as a yellow solid: m.p. 117–118°C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.69 (d, $J = 2.8$ Hz, 1H), 8.40 (d, $J = 2.8$ Hz, 1H), 4.53 (t, $J = 5.6$ Hz, 2H), 3.57 (q, $J = 5.9$ Hz, 4H), 3.19 (t, $J = 6.1$ Hz, 4H), 1.60 (s, 9 H). Analytically required for $C_{15}H_{21}N_3O_8$: C, 48.52; H, 5.7; N, 11.32. Found: C, 48.54; H, 5.8; N, 11.31.

tert-Butyl 2-(bis(2-((methylsulfonyl)oxy)ethyl)amino)-3,5-dinitrobenzoate (10)

A stirred solution of **9** (3.76 g, 10.1 mmol) in pyridine (5 ml) at 0°C was treated dropwise with MsCl (1.90 ml, 24.5 mmol), and then allowed to come to room temperature for 1 h. The mixture was diluted with water (50 ml) and the resulting solid was purified by column chromatography on silica gel, eluting with EtOAc, followed by recrystallization from EtOAc/iPr_2O to give **10** (4.79 g, 90%) as a yellow solid: m.p. 105.5–106°C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.81 (d, $J = 2.8$ Hz, 1H), 8.53 (d, $J = 2.8$ Hz, 1H), 4.30 (t, $J = 5.5$ Hz, 4H), 3.49 (t, $J = 5.5$ Hz, 4H), 3.13 (s, 6H), 1.61 (s, 9 H). Analytically required for $C_{17}H_{25}N_3O_{12}S_2$: C, 38.71; H, 4.78; N, 7.97; S, 12.16. Found: C, 38.78; H, 4.82; N, 7.96; S, 12.30.

tert-Butyl 2-((2-bromoethyl)-(2-((methylsulfonyl)oxy)ethyl)amino)-3,5-dinitrobenzoate (11)

A mixture of **10** (5.00 g, 9.48 mmol) and LiBr (0.99 g, 11.4 mmol) in DMF (10 ml) was stirred at 50°C for 1.5 h, then diluted with water and refrigerated. The precipitated semi-solid was dried and chromatographed on silica gel, eluting with EtOAc/petroleum ether (9:11) to give firstly *tert*-butyl 2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoate (1.60 g) as a yellow solid: ^{1}H NMR [$(CD_3)_2SO$] δ 8.80 (d, $J = 2.8$ Hz, 1H), 8.50 (d, $J = 2.8$ Hz, 1H), 3.61–3.54 (m, 4H), 3.54–3.47 (m, 4H), 1.61 (s, 9 H).

Later fractions gave **11** (2.11 g, 43%) as a yellow solid: m.p. (EtOAc/iPr_2O) 97–98°C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.81 (d, $J = 2.8$ Hz, 1H), 8.52 (t, $J = 2.8$ Hz, 1H), 4.29 (t, $J = 5.5$ Hz, 2H), 3.62–3.55 (m, 2H), 3.54–3.46 (m, 4H), 3.12 (s, 3H), 1.60 (s, 9 H). Analytically required for $C_{16}H_{22}BrN_3O_9S$: C, 37.51; H, 4.33; N, 8.2; Br, 15.60. Found: C, 37.65; H, 4.51; N, 8.12; Br, 15.69.

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Further elution with EtOAc gave unreacted **10** (0.92 g).

2-((2-Bromoethyl)-(2-((methylsulfonyl)oxy)ethyl) amino)-3,5-dinitrobenzoic acid (12)

A solution of **11** (2.70 g, 5.27 mmol) in TFA (12 ml) was stirred at room temperature for 3 h, then diluted with water (35 ml) to complete precipitation of the product. Recrystallization from EtOAc/iPr_2O gave **12** (2.19 g, 91%) as a yellow solid: m.p. 137–138°C. ^{1}H NMR [$(CD_3)_2SO$] δ 14.18 (br, 1H), 8.82 (d, $J = 2.8$ Hz, 1H), 8.60 (d, $J = 2.8$ Hz, 1H), 4.27 (t, $J = 5.4$ Hz, 2H), 3.59–3.46 (m, 6H), 3.12 (s, 3H). Analytically required for $C_{12}H_{14}BrN_3O_9S.0.5H_2O$: C, 30.98; H, 3.25; N, 9.03. Found: C, 32.12;32.17; H, 3.10;3.07; N, 9.18;9.23.

2-((2-Bromoethyl)-2-(((2-hydroxy(1,1,2,2-$^{2}H_4$)ethyl)-amino)carbonyl)-4,6-dinitroanilino)ethyl methanesulfonate (13)

A suspension of powdered **12** (3.12 g, 6.84 mmol) in CH_2Cl_2 (40 ml) was treated with oxalyl chloride (0.88 ml, 10.3 mmol) and DMF (1 drop) and stirred at room temperature for 1 h. Evaporation of the volatiles under reduced pressure below 25°C, followed by azeotroping with benzene gave the crude acid chloride. This was dissolved in THF (15 ml) and added dropwise to a stirred solution of 2-amino[1,1,2,2-$^{2}H_4$]ethanol (0.58 g, 8.91 mmol) (Cambridge Isotope Laboratories, Andover, MA 01810, USA) and Et_3N (1.43 ml, 10.3 mmol) in dioxane/THF (1:1) (25 ml) at 0°C. The mixture was stirred at 0°C for a further 10 min, then poured into ice-cold 0.1 N aqueous MsOH (120 ml) and extracted with EtOAc (2×80 ml). The combined organic phase was washed with water (2×40 ml), dried, and concentrated under reduced pressure. Chromatography on silica gel, eluting with EtOAc, followed by precipitation of the product from a CH_2Cl_2 solution with hexane, gave **13** (2.96 g, 86%) as a yellow gum; ^{1}H NMR [$(CD_3)_2SO$] δ 8.74 (d, $J = 2.8$ Hz, 1H), 8.71 (s, 1H), 8.35 (d, $J = 2.8$ Hz, 1H), 4.76 (s, 1H), 4.28 (t, $J = 5.5$ Hz, 2H), 3.60–3.54 (m, 2H), 3.51–3.43 (m, 4H), 3.13 (s, 3H). ^{13}C NMR [$(CD_3)_2SO$] δ 165.3, 145.8, 145.3, 140.9, 136.2, 127.5, 122.1, 67.5, 58.4 (br, CD_2), 5.43, 51.0, 41.3 (br CD_2), 36.5, 29.7. HRMS(FAB) calculated, for $C_{14}H_{16}D_4^{79}BrN_4O_9S$ (MH^+) m/z 503.0385; found, 503.0375.

HPLC analysis of the product indicated the presence of 3.4% of the corresponding chloromesylate.

2-((2-Bromoethyl)-2-((3,3,4,4-$^{2}H_4$)-6-*tert*-butoxy-8, 8-dimethyl-6-oxido-5,7-dioxa-2-aza-6-phosphanon-1-anoyl)-4,6-dinitroanilino)ethyl methanesulfonate (14)

A stirred solution of **13** (1.55 g, 3.08 mmol) and 1H-tetrazole (345 mg, 4.93 mmol) in dry DMF (8 ml) at 10°C was treated dropwise with di-*tert*-butyl diisopropylphosphoramidite (1.33 ml, 4.00 mmol, 95%). The mixture was warmed at room temperature for 2 h, then cooled to 0°C and treated slowly with a solution of 70% aqueous H_2O_2 (0.8 ml) in THF (1 ml). The mixture was warmed to 10°C for 0.5 h, then poured into ice-cold water (100 ml) and extracted with EtOAc (2×40 ml). The combined organic phases were washed with 5% aqueous $Na_2S_2O_5$ (40 ml) and water (4×40 ml), dried, and concentrated under reduced pressure below 30°C. The residue was chromatographed on silica gel, eluting with EtOAc/petroleum ether (4:1), and the eluant was concentrated under reduced pressure below 30°C to a small volume and diluted with hexane to give **14** (1.44 g, 67%) as an unstable yellow oil. ^{1}H NMR [$(CD_3)_2SO$] δ 8.92 (s, 1H), 8.76 (d, $J = 2.8$ Hz, 1H), 8.34 (d, $J = 2.8$ Hz, 1H), 4.28 (t, $J = 5.4$ Hz, 2H), 3.58 (t, $J = 7.3$ Hz, 2H), 3.51–3.43 (m, 4H), 3.13 (s, 3H), 1.43 (s, 18H).

2-((2-Bromoethyl)-2,4-dinitro-6-(((2-(phosphonooxy) (1,1,2,2-$^{2}H_4$)ethyl)-amino)carbonyl)anilino)ethyl methanesufonate (1-D4)

A stirred solution of **14** (730 mg, 1.05 mmol) in CH_2Cl_2 (5 ml) was treated with TFA (5 ml) for 1 h at room temperature, then concentrated to near dryness under reduced pressure below 30°C. The residual gum was dissolved in dry MeCN (0.5 ml) and the solution was diluted with dry CH_2Cl_2 (25 ml), filtered, and then refrigerated at 5°C for 30 h. The separated solid was collected, washed with CH_2Cl_2, and dried under high vacuum at room temperature to give **1-D4** (529 mg, 86%) as yellow crystals: m.p. 133–135°C. HPLC analysis indicated the product was 94.7% pure, with 4.7% of the corresponding chloromesylate. ^{1}H NMR [$(CD_3)_2SO$] δ 8.90 (s, 1H), 8.75 (d, $J = 2.8$ Hz, 1H), 8.37 (d, $J = 2.8$ Hz, 1H), 4.28 (t, $J = 5.4$ Hz, 2H), 3.58 (t, $J = 7.0$ Hz, 2H), 3.51–3.43 (m, 4H), 3.13 (s, 3H). $P(OH)_2$ signals not observed. ^{13}C NMR [$(CD_3OD$] δ 168.4, 147.7, 147.6, 143.5, 137.8, 128.9, 123.8, 69.2, 65.0, (br, CD_2), 56.9, 53.4, 41.0 (br, CD_2), 37.3, 29.7. HRMS(FAB) calculated for $C_{14}H_{17}D_4^{79}BrN_4O_{12}PS$ (MH^+) m/z 583.0049; found, 583.0050.

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