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SYNTHESIS OF PHOSPHONATE 3-PHTHALIDYL ESTERS AS PRODRUGS FOR POTENTIAL INTRACELLULAR DELIVERY OF PHOSPHONATES

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Abstract: A new prodrug approach for intracellular delivery of phosphonates was developed via the synthesis of 3-phthalidyl esters of 1-naphthalenemethylphosphonate. This approach is advantageous over the traditional acyloxymethyl phosphonate prodrugs, because these prodrugs do not generate formaldehyde and have improved plasma half-lives.

Phosphate and phosphonate derivatives often possess interesting enzyme inhibitory activities in vitro, and consequently are considered as potential drug candidates.[1] However, in vivo biological activities are lost due to instability, short half-life, or poor cell penetration and oral bioavailability, which constitute a major obstacle for the development of these compounds into drugs. One of the underlying problems associated with these compounds is that phosphates and phosphonates exist in ionized forms at physiological pH, thus prohibiting cell penetration via passive diffusion. A common strategy to improve cell penetration entails the conversion of these compounds to hydrophobic proesters which may penetrate cells via passive diffusion.[2] Acyloxymethyl phosphonate diesters are often used as phosphonate prodrugs, however these prodrugs tend to be chemically unstable and highly susceptible to enzymatic hydrolysis leading to short plasma half-lives.[3] Short plasma half-life of prodrugs could cause drug accumulation in the plasma as monoester and/or free acid forms limiting the intracellular delivery of these compounds. The successful use of 3-phthalidyl esters as carboxylate prodrugs for cephalosporins[4] prompted us to investigate the feasibility of 3-phthalidyl esters as phosphonate prodrugs, although carboxylate esters are generally substrates for esterases while phosphonate esters are not. To quickly determine the viability of 3-phthalidyl phosphonate esters as prodrugs, 1-naphthalenemethylphosphonate (NMPA, **1**) was selected as a model compound for organic phosphonates,[5] 3-phthalidyl esters of NMPA were synthesized, and a rapid in vitro screening assay was selected to study the intracellular activation of these esters. Herein, we report the synthesis of various 3-phthalidyl NMPA esters, the evaluations of these compounds as phosphonate prodrugs in rat hepatocytes, and the optimization of these prodrugs for enhanced plasma stability.

Chemistry. Three different synthetic strategies were used to prepare various substituted phthalides as shown in the following schemes. Using a modified Ishikawa procedure,[6] 4,7-dichlorophthalic anhydride was reduced with sodium borohydride to give 4,7-dichlorophthalide (**2b**, 46%). Alkylation of 4-hydroxy phthalic anhydride with methyl iodide followed by sodium borohydride reduction gave 4-methoxyphthalide (**2c**, 19%) and 7-methoxyphthalide (**2e**, 66%). Alternatively 4-hydroxyphthalic anhydride was reduced with sodium borohydride (70%) and subsequent alkylation with ethyl iodide gave 7-ethoxyphthalide (**2f**, 81%).

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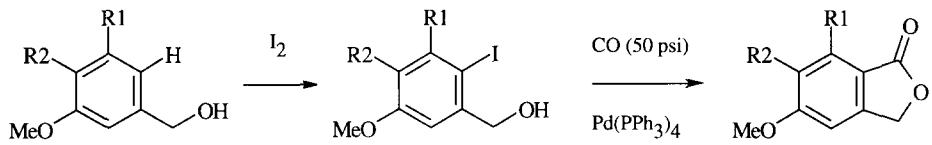
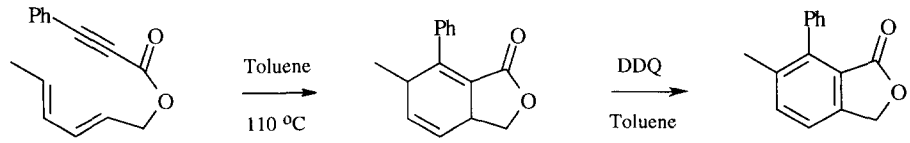
R1 = R4 = Cl, R3 = H	**2b**
R1 = R3 = H, R4 = OMe	**2c**
R1 = OMe, R3 = R4 = H	**2e**
R1 = OEt, R3 = R4 = H	**2f**

Phthalides **2d,g,h** were synthesized using a two-step procedure that was developed based on Stille's palladium catalyzed tandem carbonylation-lactonization reaction.[7] Iodination of 3-methoxybenzyl alcohols with iodine in the presence of silver trifluoroacetate gave 6-iodo-3-methoxybenzyl alcohols (**3d,g,h**), which were subjected to palladium catalyzed carbonylation - lactonization reaction to give phthalides **2d,g,h**.



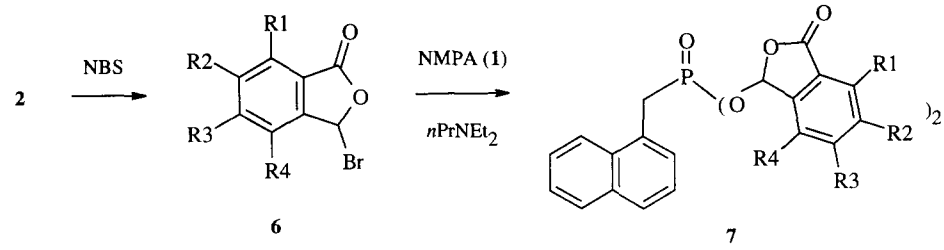
R1 = R2 = H	**3d**: 81%	R1 = R2 = H	**2d**: 82%
R1 = OMe, R2 = H	**3g**: 99%	R1 = OMe, R2 = H	**2g**: 87%
R1 = R2 = OMe	**3h**: 91%	R1 = R2 = OMe	**2h**: 77%

Phthalide **2i** was prepared via an intramolecular Diels–Alder reaction as shown below. Coupling of trans,trans-2,4-hexadiene-1-ol with phenylpropiolic acid gave compound **4** which was heated in toluene to give the intramolecular [4 + 2] cycloadduct **5** (90%), and subsequent oxidation with DDQ gave phthalide **2i** (90%).



4 **5** **2i**

3-Phthalidyl NMPA esters (**7a–i**) were synthesized via alkylation reactions between NMPA and 3-phthalidyl bromides (**6a–i**). Phthalides **2b–i** were brominated using NBS in carbon tetrachloride while being irradiated with a 250 watt lamp to give the corresponding 3-bromophthalides in good to excellent yields. NMPA was alkylated with **6b–i** in the presence of Hunig's base,[8] and isolated yields of NMPA 3-phthalidyl diesters (**7a–i**) are reported in Table 1.



Biological Testing. Results from the in vitro activation and plasma stability study of compounds **7a–h** are summarized in Table 1.

Table 1. The activation in hepatocytes and plasma stability of prodrugs (**7a–h**).

Entry	R1	R2	R3	R4	No.[a]	yield[b]	$t_{1/2}$[c]	[NMPA][d]
1					**1**		-[e]	0
2	H	H	H	H	**7a**	80%	0.5	500
3	Cl	H	H	Cl	**7b**	54%	0.1	78
4	H	H	H	MeO	**7c**	33%	-[e]	0
5	H	H	MeO	H	**7d**	30%	0.5	428
6	MeO	H	H	H	**7e**	41%	2	336
7	EtO	H	H	H	**7f**	38%	2	208
8	MeO	H	MeO	H	**7g**	50%	-[e]	359
9	MeO	MeO	MeO	H	**7h**	40%	5	246
10	Ph	Me	H	H	**7i**	92%	10	20

[a]Compound numbers. [b]Isolated yields for the conversion of NMPA (**1**) to **7a–h**. [c]$t_{1/2}$ represents half lifes (minute) in rat plasma. [d][NMPA] represents intracellular concentration (μM) of NMPA in isolated rat hepatocytes after 15 min of incubation. [e]Did not determine.

The activation of these proesters was evaluated in isolated rat hepatocytes by measuring the generation of intracellular NMPA.[9] Rat hepatocytes were isolated using standard procedures[10] and incubated in Krebs bicarbonate buffer. Suspension assays were performed at cell densities of 75 mg/mL (37 °C). Prodrugs were added as a DMSO solution (100 μM). After 15 min of incubation, cells were spun through oil into 10% perchloric acid. The cell extract was subsequently neutralized and analyzed by HPLC for NMPA. To validate this assay NMPA, NMPA-diethyl ester and NMPA-diisoBOM[11] were tested. No intracellular NMPA was detected for NMPA and NMPA-diethyl ester, while NMPA-diisoBOM (tested at 100 μM) was activated by hepatocytes generating NMPA (85 μM) intracellularly.

The plasma half-lives of these prodrugs were measured in fresh rat plasma at 37 °C.[12] At various time points following the addition of prodrugs, plasma samples were extracted with methanol and analyzed by HPLC.

Results and Discussion. The parent 3-phthalidyl NMPA diester (**7a**) efficiently generated NMPA (500 μM) in rat hepatocytes, and **7d–h** also showed good activation intracellularly with the generation of 200–400 μM of NMPA. The activation of **7b** and **7i** by rat hepatocytes was less efficient (only 20–78 μM of NMPA were generated), while **7c** was not activated at all. The SAR suggests that this phosphonate prodrug approach can tolerate various substituents at 5,6,7-positions of phthalide, while substitution at the 4-position may inhibit

enzymatic activation of prodrugs. In the plasma stability assay, NMPA-diisoBOM showed a half-life of 0.3 min,[3] and **7a** showed a similarly short plasma half-life (0.5 min). Consequently, **7b–i** were designed to study the stereo-electronic effects of substituents on the benzene ring to the plasma stability of these prodrugs. Electron-withdrawing groups are likely to make the lactone carbonyl more prone to hydrolysis, and the synthesis of **7b**, which has a decreased plasma half-life, supported this theory (entry 2, Table 1). As expected electron-donating substituents such as MeO and EtO at 5,7-positions stabilized phthalide and led to an enhanced plasma half-life (entry 6-9, Table 1), while substitution of MeO at the 6-position of phthalide did not improve plasma stability (entry 5, Table 1). Proester **7h** represents a tenfold improvement over **7a** in plasma half-life and was effective in generating NMPA (246 μM) intracellularly. The most significant improvement in plasma stability was demonstrated by **7i** ($t_{1/2}$ = 10 min) presumably due to the steric effect of the phenyl group toward the hydrolysis of the phthalide, although activation of **7i** in hepatocyte was less effective comparing to **7a**, entry 10, Table 1.

In summary, a series of 3-phthalidyl esters of NMPA were synthesized as a potential prodrug approach to improve intracellular delivery of phosphonates. These 3-phthalidyl esters successfully delivered NMPA intracellularly in rat hepatocytes, and were further optimized to give higher plasma stability. Advantages possessed by this prodrug approach over the traditional acyloxymethyl prodrug approach include that these prodrugs do not generate formaldehyde (which may be toxic)[13] and have improved plasma stability. 5,6,7-Trimethoxyphthalidyl ester of NMPA (**7h**, $t_{1/2}$ = 5 min) represents a tenfold improvement in plasma stability over the parent 3-phthalidyl prodrug (**7a**, $t_{1/2}$ = 0.5 min) and is 17-fold more stable than NMPA-diisoBOM ($t_{1/2}$ = 0.3 min) in rat plasma. Although this prodrug approach showed promise in vitro, further experiments such as solubility[14] and in vivo studies are needed to access the feasibility of this prodrug approach as a method for oral delivery of phosphonate drugs. The potential extension of this prodrug approach to phosphates are being investigated and will be reported in due course.

Acknowledgement: We thank Mr. Robert Rydzewski for the preparation of NMPA-diisoBOM.

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5. NMPA was purchased from Lancaster and used as received.

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8. General procedures for the synthesis of bis(3-phthalidyl) NMPA esters (**7a–i**): A solution of NMPA (**1**, 1 mmol) and Hunig's base (5 mmol) in anhydrous acetonitrile (4 mL) was treated with 3-bromophthalide (2.5 mmol) under nitrogen, and the resulting reaction mixture was stirred at room temperature (25 °C) for 24 h. The reaction mixture was evaporated, and the residue was purified by flash chromatography (SiO_2, 2 × 15 cm, 20, 30, 50% EtOAc-hexane, gradient elution) to give the desired product (**7a**) as a white solid (389 mg, 80%). mp 101–102 °C; ^{1}H NMR ($CDCl_3$) 8.05–7.31 (15H, m), 6.98 (1H, d, J = 8 Hz, -CH), 6.62 (1H, d, J = 8 Hz, -CH), 3.90 (2H, m, $-CH_2$). Anal. ($C_{27}H_{19}O_7P$) C, H.

Bis(4,7-dichloro-3-phthalidyl) 1-naphthalenemethylphosphonate (**7b**). Chromatography (33% EtOAc-hexane) gave a white foam (54%). ^{1}H NMR ($CDCl_3$) 7.95–7.35 (11H, m), 7.21–7.08 (2H, m, 2 × -CH), 3.95 (2H, m, $-CH_2$). Anal. ($C_{27}H_{15}O_7Cl_4P$) C, H.

Bis(4-methoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7c**). Chromatography (2% MeOH-CH_2Cl_2) gave a white powder (33%). mp 189–192 °C; ^{1}H NMR ($CDCl_3$) 8.04–7.02 (15H, m), 3.97 (3H, s, $-CH_3$), 3.89 (2H, m, $-CH_2$), 3.49 (3H, s, $-CH_3$). Anal. ($C_{29}H_{23}O_9P$) C, H.

Bis(5-methoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7d**). Chromatography (35% EtOAc-hexane) gave a white solid (30%). mp 173–177 °C; ^{1}H NMR ($CDCl_3$) 8.10–6.68 (15H, m), 3.98 (2H, m, $-CH_2$), 3.80 (6H, s, 2 × $-CH_3$). Anal. ($C_{29}H_{23}O_9P$) C, H.

Bis(7-methoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7e**). Chromatography (2% MeOH-CH_2Cl_2) gave a white solid (41%). mp 180–190 °C; ^{1}H NMR ($CDCl_3$) 8.06–6.85 (13H, m), 6.64 (1H, d, J = 8.1 Hz, -CH), 6.20 (1H, d, J = 8.1 Hz, -CH), 4.15 - 3.80 (8H, m, $-CH_2$ and 2 × $-CH_3$). Anal. ($C_{29}H_{23}O_9P + 0.25H_2O$) C, H.

Bis(7-ethoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7f**). Chromatography (50% EtOAc-hexane) gave a white solid (38%). mp 177–184 °C; ^{1}H NMR ($CDCl_3$) 8.05–6.83 (13H, m), 6.61 (1H, d, J = 8.1 Hz, -CH), 6.15 (1H, d, J = 8.1 Hz, -CH), 4.32–3.70 (6H, m, 3 × $-CH_2$), 1.60-1.46 (6H, m, 2 × $-CH_3$). Anal. ($C_{31}H_{27}O_9P$) C, H.

Bis(5,7-dimethoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7g**). Chromatography (50% EtOAc-hexane) gave a white foam (50%). ^{1}H NMR ($CDCl_3$) 8.09–5.80 (13H, m), 4.02–3.65 (14H, m, $-CH_2$ + 4 × $-CH_3$). Anal. ($C_{31}H_{27}O_{11}P + 1.67H_2O$) C, H.

Bis(5,6,7-trimethoxy-3-phthalidyl) 1-naphthalenemethylphosphonate (**7h**). Chromatography (30, 40, 50, 60, 70% EtOAc-hexane, gradient elution) gave a white solid (40 %). mp 171–175 °C; ^{1}H NMR ($CDCl_3$) 8.15–7.15 (7H, m), 6.85 (1H, d, J = 8.2 Hz), 6.65 (1H, d, J = 8.2 Hz), 6.53 (1H, s), 5.96 (1H, s), 4.19–3.64 (20H, m, $-CH_2$ and 6 × $-CH_3$). Anal. ($C_{33}H_{31}O_{13}P$) C, H.

Bis(6-methyl-7-phenyl-3-phthalidyl) 1-naphthalenemethylphosphonate (**7i**). Chromatography (25% EtOAc-hexane) gave a white solid (92%). ^{1}H NMR ($CDCl_3$) 8.14–6.90 (21H, m), 6.84 (1H, d, J = 7.8 Hz, -CH), 6.54 (1H, d, J = 7.8 Hz, -CH), 4.01-3.80 (2H, m, $-CH_2$). Anal. ($C_{41}H_{31}O_7P + 1.5H_2O$) C, H.

9. Hepatocytes were prepared from fed Sprague–Dawley rats (250–300 g) according to the procedure of Berry and Friend as modified by Groen.[10] Hepatocytes (75 mg wet weight/mL) were incubated in 1 mL Krebs-bicarbonate buffer containing 10 mM glucose and 1 mg/mL BSA. Incubations were carried out in an oxygen (95%)-carbon dioxide (5%) atmosphere in closed 50-mL Falcon tubes submerged in a rapidly shaking water bath (37 °C). Prodrugs were dissolved in DMSO to yield 10 mM stock solutions, and then diluted into the cell suspension to yield a final concentration of 100 M. After 1 h incubation, an aliquot of the suspension was removed and spun through a silicon/mineral oil layer into 10% perchloric acid. The cell extracts in the acid layers were neutralized, and the intracellular prodrug metabolite content analyzed by reverse phase HPLC using an ODS column. Gradient elution from 10 mM sodium phosphate (pH 5.5) to 75% acetonitrile was used and UV detection was set at 310 nm. Peaks on the chromatograms were identified by comparison to the retention times and spectra of prodrugs and NMPA standards.

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11. NMPA-diisoBOM: bis(isobutyryloxymethyl) 1-naphalenemethylphosphonate was prepared by NMPA alkylation with isobutyrylmethyl iodide using the procedures[8] described above.

12. Rat plasma was prepared by centrifugation of fresh blood samples obtained from the posterior vena cava of anesthetized rats. Plasma samples were incubated at 37 °C in a shaking incubator, and prodrugs added from a 10 mM DMSO stock solution to yield a final concentration of 100 M. At appropriate time points after prodrug addition, aliquots of the incubation mixtures were removed and extracted by addition of methanol to 60%. Prodrug and parent drug content was quantified by HPLC as described above. Half-lives were determined graphically by plotting prodrug concentration versus time. NMPA was stable in the rat plasma.

13. According to material safety data sheet from Aldrich, formaldehyde has an oral LD_{50} of 100 mg/kg in the rat while 2-carboxybenzaldehyde has an oral LD_{50} of 7500 mg/kg; formaldehyde is labelled as toxic, may cause cancer and heritable generic damage, while 2-carboxybenzaldehyde is labelled as irritant.

14. Although no solubility studies were conducted, these 3-phthalidyl esters appeared to have improved solubility compared to NMPA-diPOM. Because when a DMSO solution of NMPA-diPOM was added into the testing cellular medium, NMPA-diPOM oiled out and its activation could not be determined in this assay while these 3-phthalidyl esters did not present this problem. It is noteworthy that both the parent drug and the prodrug segments contribute to the actual solubility of final prodrugs. For example, NMPA is hydrophobic and therefore both NMPA-diPOM and its 3-phthalidyl esters appear to be very hydrophobic, however PMEA-diPOM has been developed into an oral drug.