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Phosphinyliminodithiolane Insecticides: Novel Addition Reactions of Phosfolan and Mephosfolan Sulfoxides and Sulfones

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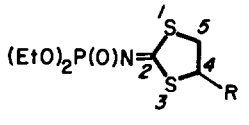
The sulfoxides and sulfones of phosfolan [2-(diethoxyphosphinylimino)-1,3-dithiolane] and mephosfolan (its 4-methyl analog) react by nucleophilic attack of water, methanol, and thiols at the imino carbon rather than at the phosphorus. Some of the addition products are in turn reactive. Thiolcarbamates formed on hydrolysis of the sulfoxides and sulfones further react with water at physiological pH giving diethyl phosphoramidate via a carbamic acid and with methanol yielding a methyl carbamate. Sulfenic acids from sulfoxide addition reactions readily condense with thiols, e.g., phosfolan sulfoxide readily forms a bis conjugate from addition of two molecules of glutathione. These addition reactions may serve as models for inhibition of acetylcholinesterase, detoxification, and tissue binding.

INTRODUCTION

Organophosphorus (OP) toxicants generally act by phosphorylation of acetylcholinesterase (AChE) at its active-site serine hydroxyl (*1*). The OP insecticides phosfolan (**1**) and mephosfolan (**2**) are unusual in that they lack a "classical" acyl moiety or leaving group for enzyme phosphorylation (*2*). However, **1** and **2** become 160- to 47,000-fold more potent AChE inhibitors on microsomal oxidative bioactivation and peracid oxidation to their sulfoxides and sulfones (**3–7**) (*3*). It is not clear if the *S*-oxide activation products act as AChE inhibitors by phosphorylation or another type of reaction. Nucleophiles may serve as models of the AChE active-site serine hydroxyl and cellular sites involved in hydrolytic and conjugative detoxification (*2, 4*). This study examines the addition reactions of **3–7** with water, thiols, and methanol as potential biomimetic models.



1 phosfolan (R=H)
2 mephosfolan (R=CH_3)
3 phosfolan sulfoxide
4 phosfolan sulfone
5 mephosfolan 3-sulfoxide
6 mephosfolan 3-sulfone
7 mephosfolan 1-sulfone

0045-2068/85 $3.00

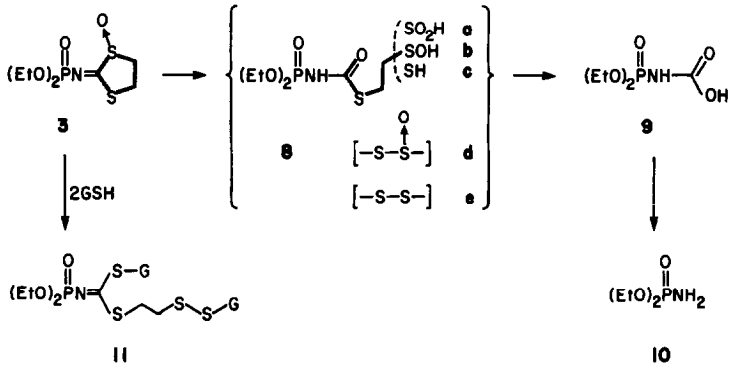


FIG. 1. Addition reactions of phosfolan sulfoxide (3) (GSH, glutathione).

RESULTS

Hydrolysis of Phosfolan and Mephosfolan

Iminodithiolanes **1** and **2** are stable for 24 h in 50 mM, pD 7.4, Hepes buffer, but **1** undergoes ~20% hydrolysis in 1 h in 1.5 M NaOD to give diethyl phosphate as the only phosphorus-containing product.

Phosfolan Sulfoxide Addition Reactions (Fig. 1)

Sulfoxide **3** hydrolyzes slowly in water and at pD 5 but is completely hydrolyzed at pD 6 in 80 min and at pD 8.3 in 2 min, each at 20°C. Within 15 min at pD 6, **3** gives **9** and **10** plus a broad NMR region designated as **8b–e** (Fig. 2). Kinetic studies indicate the pathway **3** → **8** → **9** → **10**. Formation of **9** is accompanied by liberation of a thiol fragment that precipitates from solution. Identification of the

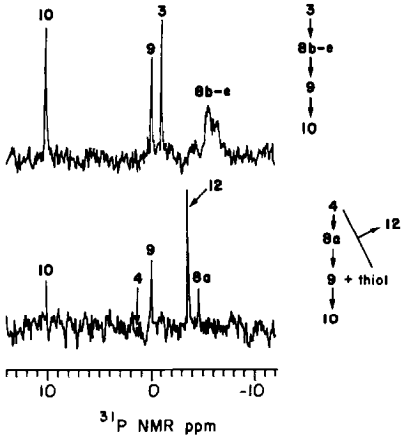


FIG. 2. ^{31}P NMR spectra of hydrolysis products of phosfolan sulfoxide (3) and sulfone (4) after 15 min incubation in pD 6, 0.5 M, citrate buffer at 20°C.

TABLE 1

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MASS SPECTROMETRY DATA FOR PHOSFOLAN AND MEPHOSFOLAN S-OXIDE ADDITION PRODUCTS

Compound	^{1}H NMR[a]	^{13}C NMR[a] of CN	^{31}P NMR[b]	MS[c]
8a[d]	3.09 (t), J = 6.9 (Hb, Hc); 3.25 (t), J = 6.9 (H, Ha); 8.5 (d), J = 14 (NH)	168.1	−5.3	306 $[MH]^+$
9	—	—	−0.2	—
10	—	—	10.0	154 $[MH]^+$
11	See Fig. 3	185.2	−4.1	868 $[MH]^+$
12	3.08 (t), J = 7.5 (Hb, Hc); 3.52 (t), J = 7.5 (H, Ha); 3.94 (s) (OCH_3)	175.4	−3.9	412 $[MH]^-$
13[e]	3.07 (t), J = 6.2 (H, Ha); 3.33 (t), J = 6.2 (Hb, Hc); 3.94 (s) (OCH_3)	171.2	−1.4	320 $[MH]^+$
14	3.62 (s) (OCH_3); 7.6 (d), J = 14 (NH)	153.9	−5.0	212 $[MH]^+$
15	1.28 (d), J = 6 (CH_3); 2.98 (ddd), J = 14, 9, 5 (Ha); 3.29 (dd), J = 14, 9 (Hb); 3.71 (dd), J = 14, 5 (Hc)	175.8	−3.9	440 $[MH]^-$

[a] —SCHbHcCRHaSO_2H of **8a**, **12**, **13**, and **15** where R=H or CH_3. Hb/Hc and R/Ha assignments are based on ^{13}C/^{1}H correlated NMR with the downfield carbon being nearest —SO_2H. NMR chemical shifts (δ ppm) and coupling constants [J (in Hz)], **8a**, **13**, and **14** in $CDCl_3$, others in D_2O; ^{1}H, 300 MHz ($CDCl_3$ δ 7.25, HDO δ 4.8); ^{13}C, 75 MHz ($CDCl_3$ δ 77, acetone carbonyl in D_2O δ 206).

[b] ^{31}P NMR in D_2O except **13** and **14** in $CDCl_3$; ^{31}P, 121.5 MHz (external 1% trimethyl phosphate in D_2O or $CDCl_3$ δ 0.0).

[c] Quaismolecular ion $[MH]^+$ by chemical ionization (methane) (**10** and **14**) or positive ion FAB (**8a**, **11**, and **13**), or $[MH]^-$ by negative ion FAB (**12** and **15**).

[d] ir, C=O, 1685 cm^{-1} (film).

[e] ir, C=N, 1605 cm^{-1} (KBr).

thiolcarbamates is based on similar chemical and spectroscopic properties to **8a** (Table 1), including formation and stability at acid pD (1% DCl) and ^{31}P NMR shifts (δ −5.6 to −5.7) and MS showing a number of quasimolecular ions (M + 23 with sodium, +1 with D_2O) that are consistent with **8a**, **8b**, **8c**, and **8e**. Compounds **9** and **10** are identified as the carbamic acid and amide, respectively, by comparison with standards.

Sulfoxide **3** hydrolyzes to **10** in pD 7.4, 50 mM, Hepes buffer, but in pD 7.0, 0.5 M, Hepes buffer ~30% hydrolyzes to **10** with the remainder forming several new but unidentified products (^{31}P NMR of major products δ −0.5 to −0.6, pD 7). Diethyl phosphoric acid is not formed on hydrolysis of **3** under any of the conditions examined.

The glutathione (GSH) conjugate **11** is the only phosphorus-containing product formed on treatment of **3** with 2.5 equivalents of GSH at pD 8.3 (Fig. 1). Purified **11** is identified by MS (Table 1) and NMR. In a ^{1}H/^{1}H correlated (COSY) experiment (Fig. 3) the 12′,12″ and 7′,7″ positions are clearly distinguishable, but positions further from the attachment sites are unresolved as expected. The absence of detectable long-range ^{13}C/^{1}H couplings precluded the direct assignment of 12′,7′ relative to 12″,7″ and 4 relative to 5. This was achieved indirectly by com-

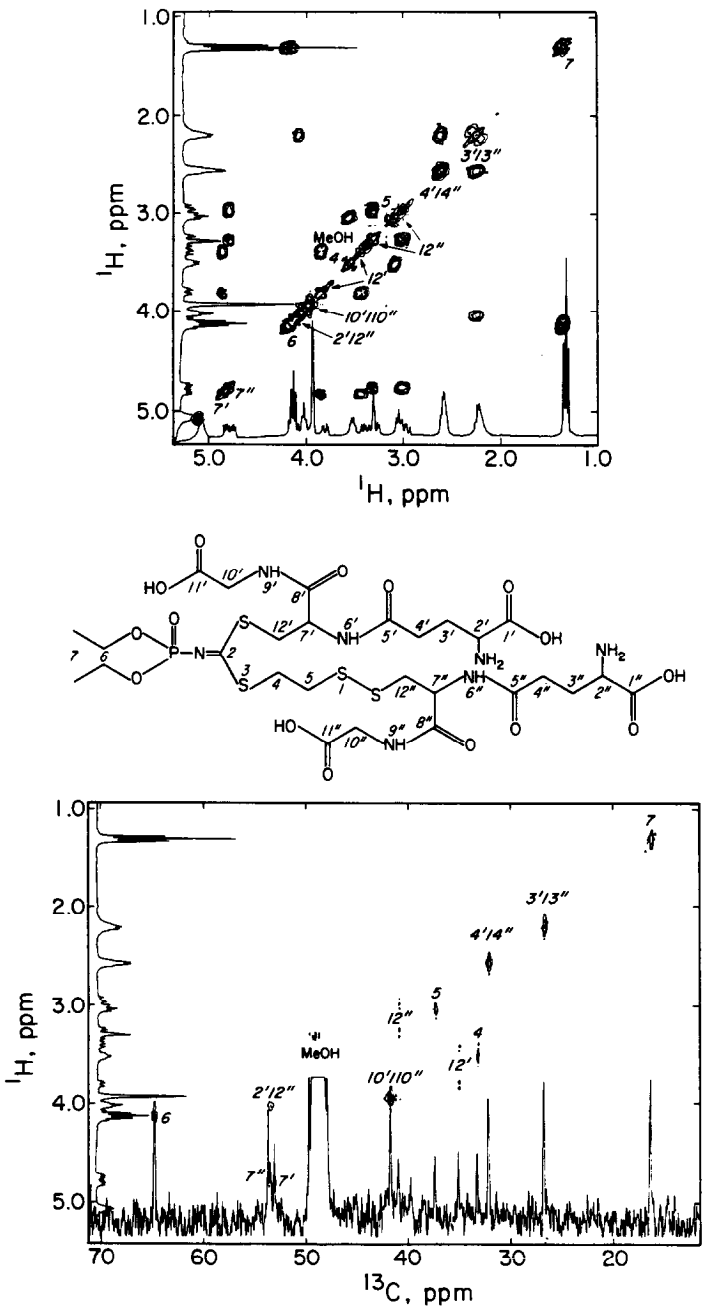


FIG. 3. ^{1}H/^{1}H correlated (COSY) and ^{13}C/^{1}H correlated NMR spectra identifying **11** along with the data in Table 1 (standard Bruker Software).

parison of a ^{13}C/^{1}H correlated NMR spectrum of **11** (Fig. 3) to a similar spectrum of GSH disulfide (GSSG). The α- and β-cysteinyl positions in GSSG have ^{1}H and ^{13}C shifts coincidental with the positions in **11** assigned here as 12" and 7". The 4

and 5 positions are tentatively assigned on the expectation of similar shielding at the 4 and 12′ positions and at the 5 and 12″ positions. The $^{13}C/^{1}H$ experiment shows an interesting interchange between the ^{1}H and ^{13}C spectra in that in the case of the positions discussed above the upfield position in the ^{1}H spectrum corresponds to the more deshielded position in the ^{13}C spectrum.

Phosfolan Sulfone Addition Reactions (Fig. 4)

Sulfone **4** is much less stable than sulfoxide **3** in buffered media at pD 5 and above. At pD 6, **4** completely reacts within 15 min at 20°C to give **12** (stable under these conditions) and **8a** which further hydrolyzes to **10** via the transient intermediate **9** (Fig. 2). As observed with **3**, sulfone **4** and thiolcarbamate **8a** are progressively less stable at pD 5, 6, and 8.3, e.g., no **4** is detected after 16, 7, and 2 min, respectively, and no **8a** after 15 h, 30 min, and 2 min, respectively. In opposing fashion, **9** is increasingly more stable at pD 5, 6, and 8.3, e.g., no **9** is detected at pD 5 or after 30 min at pD 6, whereas only ~50% decomposes to **10** in 80 min at pD 8.3. The hydrolysis sequence is **4** → **8** → **9** → **10**, analogous to that for **3** except that **4** also adds mercaptoethanesulfinic acid giving **12** (identified according to Table 1) at pD 5 and above. This reaction is faster at increasing pD up to 8.3 where the yield approaches the 50% theoretical maximum. Thus, hydrolysis of **4** differs from **3** in having more rapid NC—S(O_n) bond cleavage giving a single thiolcarbamate **8a** versus many thiolcarbamates **8a–e** and in also having a competing addition reaction giving **12**.

Sulfone **4** hydrolyzes to sulfinic acids in pD 7.4, 50 mM, Hepes buffer, lowering the buffer pD and giving **8a**, **10**, **12**, and a new unidentified compound (δ −0.3, pD 8) as stable products. The new compound is the only product detected from **4** in 0.5 M, Hepes buffer at pD 7 or 8.

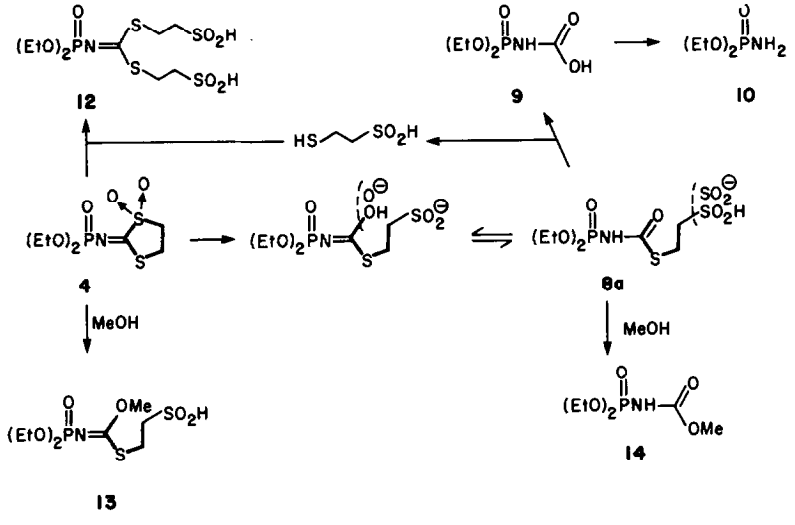


FIG. 4. Addition reactions of phosfolan sulfone (4).

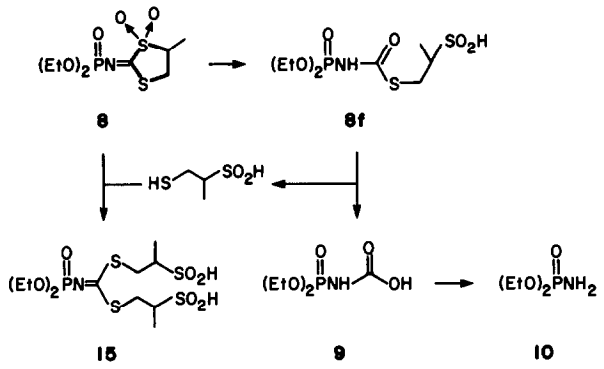


FIG. 5. Addition reactions of mephosfolan 3-sulfone (**6**).

Methanol adds to **4** and **8a** giving exclusively **13** and **14**, respectively (identified according to Table 1).

Addition Reactions of Mephosfolan S-Oxides (**5–7**)

Sulfoxide **5**, as with **3**, gives only **10** in pD 7.4, 50 mM, Hepes buffer. 3-Sulfone **6** in pD 8.3, $NaHCO_3$ buffer gives **15** and **10** via **9** and presumably **8f** (Fig. 5) in similar fashion to **4**. In pD 7.4, 50 mM, Hepes buffer, formation of sulfinic acids lowers the buffer pD stabilizing **8f** in addition to **10** and **15**. Identification of **8f** is based on its stability at acid pD and ^{31}P NMR shift and **15** is identified according to Table 1. In pD 7.4, 50 mM, Hepes buffer, **7** also lowers the buffer pD and ^{31}P NMR shows shifts expected of **10**, a thiolcarbamate, a thiol addition product, and a product corresponding to the unknown from **4** in Hepes buffer.

DISCUSSION

Phosphinyliminodithiolanes **1** and **2** are much more stable than sulfoxides **3** and **5** and especially sulfones **4**, **6**, and **7**, which are very susceptible to nucleophilic attack at their imino carbon. The sulfoxides undergo addition at the imino carbon with water (**3** → **8a–e**) and a thiol such as GSH (**3** → **11**) and the sulfones similarly react with water (**4** → **8a**), thiols (**4** → **12** and **6** → **15**), and methanol (**4** → **13**). Hepes addition to most or all of the *S*-oxides, more completely to the sulfones than the sulfoxides, presumably results from a similar addition at the imino carbon (**3–7** → **16**) (Fig. 6). The more rapid hydrolysis of **4** than of **3** is expected based on the greater acidity of sulfinic than sulfenic acids (*5–7*) and the enhanced reactivity of the sulfones may explain in part their greater potencies (*3*). The exceptional reactivity of the phosphinyliminodithiolane *S*-oxides relative to other sulfoxides and sulfones (*5, 6, 8*) may be attributable to formation of stabilized addition intermediates such as **18** which permit greater electron delocalization by nitrogen as compared to a similar dithiolane sulfoxide that lacks the nitrogen (*9*). The excep-

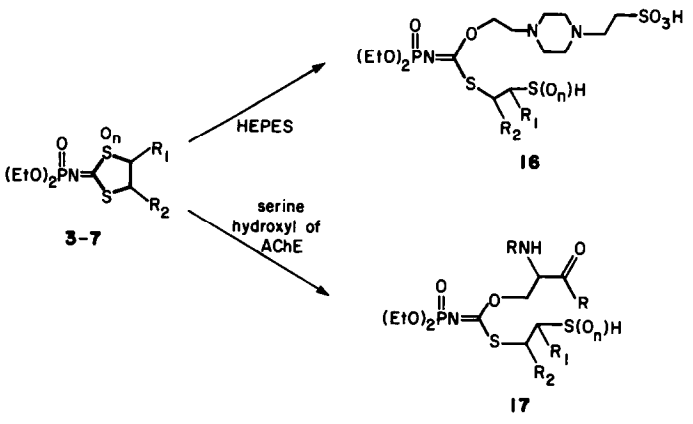
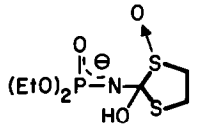


FIG. 6. Possible addition reactions of phosphinyliminodithiolane sulfoxides and sulfones (3–7) to Hepes buffer and AChE serine hydroxyl.

tionally high reactivity of the sulfoxides may be analogous to that of β-sulfinyl enones (*10*).



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These addition reactions (especially methanolysis, **4** → **13**) establish a potential precedent for the mechanism of AChE inhibition (**17**, Fig. 6). Addition to an imino carbon as suggested here has not previously been reported as a mechanism of AChE inhibition or to our knowledge as a mechanism for any other suicide enzyme inhibition reaction.

The sulfinic acids generated from sulfone cleavage do not undergo further reactions at the liberated sulfinate group but the sulfenic acids from sulfoxide cleavage condense giving thiosulfinates and apparently a variety of other products (**8b** → **8d** → **8a**, **8c** and **8e**) expected from hydrolysis of thiolsulfinates (*7, 11*). Sulfenic acids are especially reactive with thiols (e.g., **3** → **11**, also involving imino carbon addition), a reaction used to trap sulfenic acids as disulfides (*7, 11, 12*). Apparently self-condensation of mercaptoethanesulfenic acid is more favorable than addition of the thiol to the imino carbon of **3** disfavoring a reaction parallel to **12** from **4** and **15** from **6** and instead forming a precipitate that probably is a 1,2-ethanedisulfide polymer. Biological addition reactions at the imino carbon presumably lead secondarily to disulfides with GSH and other thiols by parallel processes. These reactions would account for a previous observation that **2** is an irreversible inhibitor of mixed-function oxidases and that a metabolite binds to the protein through disulfide linkages and possibly also at the imino carbon (*13*).

Thiolcarbamates (e.g., **8a–e**) are reactive intermediates generated on hydrolysis of **3–7**. They undergo nucleophilic attack at the carbonyl carbon by water (**8a** →

9), at rates increasing with pH, and by methanol (**8a** → **14**). Base-catalyzed conversion of **8** to **9** appears to be a standard $B_{AC}2$ hydrolysis rather than proceeding via a reactive isocyanate (as with aryl carbamates) (*14*). Although **9** is relatively stable in base, it decomposes to **10** (as expected) (*14*), at an increasing rate as the pH is decreased. The thiolcarbamates may undergo parallel reactions in biological systems giving carbamoylation of the serine hydroxyl at the AChE active site; however, this is probably not important in the action of **1** and **2** since **8a** is a poor AChE inhibitor relative to **3–7** (*3*).

Hydrolysis is a potentially important detoxification mechanism because it occurs rapidly at physiological pH with **3–7** → **8** → **9** → **10** giving less potent AChE inhibitors (*3*). The facile formation of **11** suggests that GSH conjugation *in vivo* may detoxify the sulfoxides and formation of **12** and **15** indicates possible addition of GSH to the imino carbon of the sulfones. Formation of **12** and **15** per se probably does not occur *in vivo* due to low concentrations of the required thiols. The unexpected addition of Hepes (**16**) shows the high reactivity of the *S*-oxides, but microsomal activation and AChE inhibition by the *S*-oxides in phosphate versus Hepes buffers show that this reaction does not compete effectively to alter the reported anti-AChE potencies (*3*).

EXPERIMENTAL

Phosphinyliminodithiolanes **1–7** and products **8a**, **10**, and diethyl phosphate were obtained or synthesized as previously reported (*3*). New compounds (Figs. 1, 4, and 5) were identified by NMR and MS (Table 1) [for conditions see Ref. (*3*)]. GSH adduct **11** was prepared in ~40% yield by treating **1** (0.4 mmol) with *m*-chloroperbenzoic acid (0.3 mmol) in chloroform (1.5 ml) for 30 min at 20°C, and then shaking this mixture with GSH (0.6 mmol) in water saturated with $NaHCO_3$ (1 ml) and purifying the adduct from the acidified water phase by HPLC [PRP-1 C_{18} column, Rainin Instrument Co., Woburn, Mass.; methanol–0.1% trifluoroacetic acid (TFA) in water, 0.54 : 1]. Disulfinic acids **12** and **15** were obtained in ~40% yield by treating **4** and **6** (0.1 mmol) with 0.5 M, $NaHCO_3$ (1 ml) for 1 h before acidifying with TFA and purifying by HPLC (PRP-1 C_{18} column, methanol–0.05% TFA in water, 0.8 : 1 for **12** and 1 : 1 for **15**). Treatment of **10** with oxalyl chloride produced diethyl phosphoroisocyanate (*15*), which gave **9** in bicarbonate and **14** in methanol. Similarly, **8a** (0.1 mmol) in methanol (1 ml) for 12 h at 20°C gave **14** as the only phosphorus-containing product. Reflux of **4** (0.1 mmol) for 6 h in methanol (10 ml) quantitatively gave **13**. Hydrolysis rates and products were determined by NMR at 20°C with 10 to 50 mM solutions in pD 5 and 6, 0.5 M, citrate buffer and pD 8.3, 0.5 M, $NaHCO_3$ buffer and Hepes buffer as specified. Other tests with pD 7 to 9, 0.2 M, borate buffer gave results similar to citate and $NaHCO_3$, thus corroborating the uniqueness of the Hepes products.

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