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The Cleavage of RNA Model Compounds: The Interplay Between the Nucleophile and the Leaving Group

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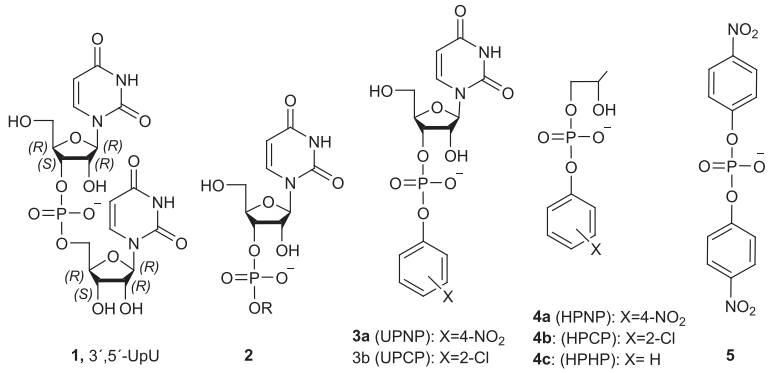
ABSTRACT

Hydrolytic reactions of phosphodiester bonds of RNA have been extensively studied over several decades. Information on the factors that affect the reactivity of phosphodiester bonds in biomolecules is important for the development of new nucleic acid-related therapeutics. Furthermore, the development of artificial nucleases requires efficient catalytic entities, and rational design of catalysts requires detailed understanding of the catalytic mechanisms. In the present article, we concentrate on the interplay between the nucleophile and leaving group both in the absence and in the presence of metal ion catalysts. The effect of the nucleophile on the reactivity of RNA model compounds has been studied with 2-hydroxypropyl and uridine 3′-aryl phosphates as well as with bis-(*p*-nitrophenyl)phosphate as substrates. pH-rate profiles for three different 2-hydroxypropyl arylphosphates were compared with those obtained with a uridine 3′-alkyl and aryl phosphates. The observations are discussed in terms of the relative goodness/poorness of the nucleophile and the leaving group. Metal complex-dependent reactions were studied in the presence of well-known and robust CuTerPy and CuBiPy complexes. The results show that CuTerPy and CuBiPy favour different types of phosphodiesters as substrates, depending on the properties of the nucleophile and leaving group, and suggest that the complexes utilize different catalysis mechanisms, which may depend also on the structure of the substrate. The results obtained further the understanding on the basic principles of metal complex-promoted cleavage of RNA and model compounds, help to assess the relevance of data obtained with model compounds and support the design of artificial enzymes for phosphodiester cleavage.

1 | Introduction

Transesterification reactions of phosphodiester bonds have been extensively studied over several decades [1, 2]. The original motivation was to study the details of the catalysis by RNA cleaving enzymes, and the discovery of ribozymes in the 1980s further intensified the interest in studies with simple RNA models [3, 4]. These days, the stability of RNA is an essential factor in the development of RNA-based therapeutic methods [5–7], and the recognition of factors that affect the reactivity is of utmost importance. Development of artificial nucleases is another important reason for studies on the reactivity of RNA models, and numerous papers reporting on the design, synthesis and catalytic properties of different types of catalysts have been published [8–13]. Dinucleoside monophosphates, such as uridylyl-3′,5′-uridine (3′,5′-UpU, **1**), nucleoside 3′-alkyl (**2**)

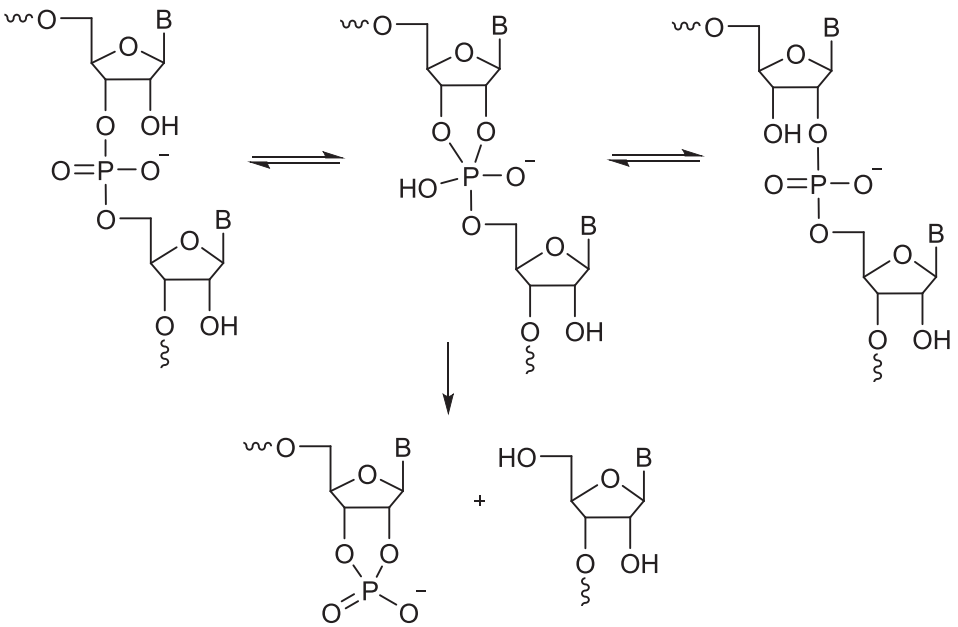
and arylphosphates (**3**) to non-nucleosidic compounds, such as 2-hydroxypropyl arylphosphates (**4**), have been used as model compounds to screen the catalytic efficiency and to study the reaction and catalysis mechanisms [1].



The cleavage of a phosphodiester bond of RNA is a transesterification reaction, where an intramolecular nucleophile replaces the leaving group (Scheme 1) [14]. If the nucleophile and the leaving group are described qualitatively as good or poor, the leaving group in RNA is poor; a pK_a value of 14.4 at 90°C has been kinetically determined for the 5′-OH leaving group in the cleavage of 3′,5′-UpU (**1**) [15]. The 2′-OH of a nucleoside can be regarded as a good nucleophile as the structure places it in a position that is favourable for the nucleophilic attack on the phosphate [16, 17]. Consequently, the departure of the leaving group is the rate-limiting step of the cleavage of RNA.

We have been studying systematically the mechanism of RNA cleavage in the absence and in the presence of metal ion-based catalysts and have previously published a paper on the effect of the leaving group acidity on the rate enhancement obtained by various metal ion complexes [18]. The results obtained with uridine 3′-alkylphosphates (**2**) showed that the weakest catalysis was observed with substrates with the best leaving groups. Generally, the catalytic activity increased as the leaving group became poorer, but there were also differences between catalysts. With nucleoside 3′-aryl phosphates (**3**), the opposite was true, and the catalytic activity generally increased as the leaving group became better.

In previous studies, the nucleophile in the reaction was the same, the 2′-OH of the ribose, whereas the leaving group varied from aryl groups (pK_a 7–10) to alkyl groups (pK_a 12–16) [18]. In the present paper, we concentrate on the combined effect of different nucleophiles and good leaving groups. Although the effect of the leaving group can be systematically studied using a series of different phosphodiesters [13, 18–21], corresponding studies on the effect of the nucleophile are much more complicated, and only a few papers have been published [22–25]. In their elegant approach, Ye et al. [22] have synthesized RNA molecules with an extra substituent at C2′ of the ribose thus altering the pK_a of the 2′-OH nucleophile. A different approach has been utilized by Bonfá et al. [24] and Livieri et al. [25], who studied the effect of the metal-activated

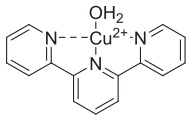


SCHEME 1 | Transesterification reactions of an RNA phosphodiester bond.

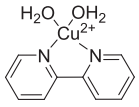
nucleophile on the cleavage of 2-hydroxylpropyl *p*-nitrophenyl phosphate (HPNP 4**a**) and bis-(*p*-nitrophenyl phosphate (BPNP, **5**) by determining rate constants for the cleavage as a function of the pK_a value of the metal-bound aquo ligand or the 2-OH group of HPNP.

Our approach is less ambitious, and we aim at a qualitative understanding on the effect of nucleophile's properties, and on the interplay between the nucleophile and the leaving group in the absence and in the presence of simple metal catalysts. The first part of the article studies the effect of pH on the reactivity of different types of RNA model compounds in the absence of metal ion catalysts. The reactivity of 2-hydroxypropyl phosphates with different aryl leaving groups (**4a–c**) was studied first. HPNP (**4a**) that is often used as a simple RNA model, is, in a sense, a mirror image of RNA with a poor nucleophile and a good leaving group. The flexible structure of the 2-hydroxypropyl chain disfavours the nucleophilic attack in comparison with a nucleophilic attack by the ribose 2′-OH group in RNA, whereas the 4-nitrophenol with a pK_a of 7.14 [19] is by far a better leaving group than a nucleoside. Two other 2-hydroxypropyl arylphosphates, 2-hydroxypropyl *o*-chlorophenyl phosphate (HPCP, **4b**) and 2-hydroxypropyl phenyl phosphate (HPHP, **4c**), contain the same flexible nucleophile, but poorer leaving groups with pK_as of 8.48 and 9.95, respectively [19]. The next step was to compare three different substrates forming two pairs: one sharing the same good nucleophile and the other with the same good leaving group.

In the second part of the article, the cleavage of three different model compounds, uridine 3′-*p*-nitrophenylphoshate (**3a**, UPNP), **4a** (HPNP) and **5** (BPNP), with the same 4-nitrophenol leaving group, and three different nucleophiles, 2′-OH of ribose, an intramolecular 2-OH of 2-hydroxypropyl moiety and an external H_2O/HO^-, respectively, were studied in the presence of Cu^{2+}-terpyridine (CuTerPy) and Cu^{2+}-bipyridine (CuBiPy) complexes. CuTerPy and CuBiPy were chosen because they are robust complexes, and their structure and properties are well known [26–31]. They have also been used as an RNA and DNA cleaving agent in various constructs [7, 32–35].



Cu^{2+}-terpyridine (CuTerPy)



Cu^{2+}-bipyridine (CuBiPy)

2 | Experimental Methods

2.1 | Kinetic Experiments

The pH of the reaction solutions was adjusted using aqueous HCl and NaOH under acidic and basic conditions, respectively. pH between values 3 and 10 was adjusted with 0.1 M formic acid, acetic acid, MOBS (4-[*N*-morpholino]butanesulfonic acid) and CHES (2-[cyclohexylamino]ethanesulfonic acid) buffers. Ionic strength was adjusted to 0.1 M with NaCl. CuTerPy and CuBiPy catalysed reactions were carried out in 50 mM MOPSO (3-morpholino-hydroxypropanesulfonic acid) or MOBS buffer solutions. Cu complexes were added as 50 mM stock solutions that were prepared using analytical grade reagents. In experiments with Cu complexes, $NaNO_3$ was used to adjust the ionic strength of reaction solutions. All pH values of reaction solutions refer to the temperature of the given experiment. The pK_a values of MOBS [36] and MOPSO [37] at elevated temperatures have been reported before. pH values were checked with a pH meter at room temperature.

Reactions were carried out in stoppered tubes in a water bath or in Eppendorf tubes in dry block heater. Aliquots of 25 to 100 μL were withdrawn, and they were cooled down on an ice bath. Acid- and base-catalysed reactions were quenched with concentrated general base or acid solution, respectively. EDTA solution was used to quench CuTerPy and CuBiPy catalysed reactions. Typically, the number of aliquots was 10–12, and the timing was planned to cover approximately two half-lives.

Aliquots were analysed by RP-HPLC using an Aquasil C-18 column (150x4 mm, particle size 5 μm). Eluents were mixtures of acetate buffer (50 mM, pH 4.3, ionic strength 0.1 M) and HPLC-grade acetonitrile. Reaction components were detected with a UV detector at the wavelength of 260, 270 and 280 nM for HPHP (**4c**), HPCP (**4b**) and HPNP (**4a**) as well as other *p*-nitrophenyl phosphodiesters, respectively. Rate constants were calculated from the disappearance of the starting material using the integrated first-order rate law.

2.2 | Synthesis and Characterization of 2-Hydroxypropyl Arylphosphates

2-Hydroxypropyl phosphates **4a–c** as a racemic mixture of two enantiomers were prepared according to a procedure reported in Brown and Usher [38] and characterized by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The spectra are available in Supporting information S1. The synthesis of **3a** has been reported in Korhonen et al. [18]. Bis-(*p*-nitrophenyl)phosphate (**5**) was a product of Sigma Aldrich.

2-Hydroxypropyl *p*-nitrophenyl phosphate **4a**: ^{1}H-NMR (500 MHz, D_2O): δH 8.20 (2H, d, J = 9.15 Hz), 7.30 (2H, d, J = 9.10 Hz), 3.98–3.93 (1H, m), 3.90–3.86 (1H, m), 3.78–3.73 (1H, m), 1.09 (3H, d, J = 6.45 Hz) ppm. ^{13}C-NMR (126 MHz, D_2O): δC = 157.4, 143.5, 125.8, 120.4, 70.0, 66.5, 17.6 ppm. ^{31}P-NMR (202 MHz, D_2O): δP −4.8 ppm.

2-Hydroxypropyl *o*-chlorophenyl phosphate **4b**: ^{1}H-NMR (500 MHz, D_2O): δH 7.23 (1H, d, J = 8.05 Hz), 7.10–7.11 (1H, td, J = 1.3, 2.55, 8.2 Hz), 7.03–7.06 (1H, dt, J = 1.55, 7.5, 15.6 Hz), 6.89 (1H, t, J = 7.8, 15.2 Hz), 3.68–3.77 (2H, m), 3.56–3.61 (1H, m), 1.64 (1H, s), 0.90 (3H, d, J = 6.45 Hz) ppm. ^{13}C-NMR (126 MHz, D_2O): δC = 147.6, 130.3, 128.1, 125.2, 125.1, 122.4, 71.0, 67.9, 17.9 ppm. ^{31}P-NMR (202 MHz, D_2O): δP −4.1 ppm.

2-Hydroxypropyl phenyl phosphate **4c**: ^{1}H-NMR (500 MHz, D_2O): δH = 7.34 (2H, t, J = 7.95, 7.85 Hz), 7.15 (3H, t, *p*-H5, J = 4.25, 3.9 Hz), 3.96–3.84 (2H, m), 3.76–3.72 (1H, m), 1.10 (3H, d, J = 6.45 Hz) ppm. ^{13}C-NMR (126 MHz, D_2O): δC = 151.7, 129.7, 124.3, 120.2, 70.8, 66.7, 17.6 ppm. ^{31}P-NMR (202 MHz, D_2O): δP −3.9 ppm.

3 | Results and Discussion

3.1 | The Reactivity of 2-Hydroxypropyl Phosphoesters 4a–c in the Absence of Metal ion Catalysts

The cleavage of 2-hydroxylpropyl *p*-nitrophenyl phosphate (HPNP, **4a**), 2-hydroxylpropyl *o*-chlorophenyl phosphate (HPCP, 4**b**) and 2-hydroxylpropyl phenyl phosphate (HPHP, 4**c**) was studied over a wide pH-range at 90°C. Under these conditions, the transesterification resulting in the release of phenol, or a substituted phenol, was the only reaction observed. The pH-rate profiles for the transesterification of **4a–c** are shown in Figure 1. The basic shape of the pH-rate profiles is similar for all three substrates: both acid and base catalyse the reaction, and the reactivity minimum is reached around pH 5. Above pH 7, the reaction of all three substrates shows a first-order dependence on hydroxide ion concentration. The dependence on acid concentration is steeper, and a second-order dependence on acid concentration over a narrow pH range is clearly observed with the less reactive substrates HPCP (**4b**) and HPHP (**4c**), and less clearly with HPNP (**4a**). The shape of the pH-rate profiles also closely resembles the shape of profiles determined for nucleoside-based RNA models such as 3′,5′-UpU (**1**) [39] and nucleoside 3′-alkyl phosphates (**2**) with different alkyl leaving groups [40].

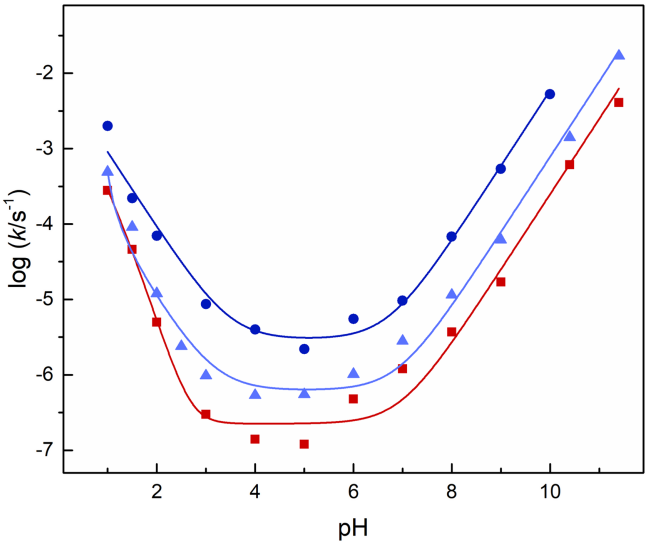


FIGURE 1 | pH-rate profiles for the cleavage of 2-hydroxypropyl phosphodiesters **4a–c.** Notation: blue circles: HPNP (**4a**); light blue triangles: HPCP (**4b**); red squares: HPHP (**4c**).

The reactivity differences between the 2-hydroxypropyl phosphodiesters **4a–c** are rather modest and remain almost the same over the pH range studied, apart from the very acidic conditions, where the difference clearly decreases. The observation is shown more quantitatively by β_{LG} values, which for the 2-hydroxypropyl esters remain practically the same over the pH range 3–10 at 90°C, as shown by the values collected in Table 1. Values of −0.4 to −0.5 are well consistent with a value of −0.56 obtained at a lower temperature for a wide range of 2-hydroxypropyl phosphates [38].

The β_{LG} values obtained for 2-hydroxypropyl phosphates **4a–c** are rather similar also to those obtained for uridine 3′-arylphosphates (**3**), at least for acid- and base-catalysed reactions. Davis, Hall, and Williams [19] have reported values of −0.54 and −0.59 for specific and general base catalysed reaction of uridine 3′-arylphosphates (**3**) at 50°C, and values of −0.27 and −0.2 have been reported at 90°C in 0.1 M HCl

TABLE 1 | β_{LG} values for the cleavage of RNA model compounds in the absence of metal ion catalysts.

Substrates	Conditions/reaction	β_{LG}
2-Hydroxypropyl arylphosphates **4a–c**	pH 1.0, 90°C	−0.30 ± 0.01[a]
2-Hydroxypropyl arylphosphates **4a–c**	pH 4.0, 90°C	−0.4 ± 0.1[a]
2-Hydroxypropyl arylphosphates **4a–c**	pH 6.0, 90°C	−0.4 ± 0.1[a]
2-Hydroxypropyl arylphosphates **4a–c**	pH 8.0, 90°C	−0.45 ± 0.07[a]
2-Hydroxypropyl arylphosphates **4a–c**	10 mM NaOH, 60°C	
Uridine 3′-arylphosphates (**3**)	Specific base catalysed, 50°C	−0.54[b]
Uridine 3′-arylphosphates (**3**)	General base catalysed, 50°C	−0.59[b]
Uridine 3′-arylphosphates (**3**)	0.1 M HCl, 90°C	−0.27[c]
Uridine 3′-arylphosphates (**3**)	0.1 M HCOOH buffer, pH 3.5, 90°C	−0.2[c]
2-Hydroxypropyl arylphosphates	50 mM NaOH, 80°C	−0.56[d]
Uridine 3′-alkylphosphates (**2**)	HO^- catalysed cleavage, 90°C	−1.28[e]
Uridine 3′-alkylphosphates (**2**)	H_3O^+ catalysed cleavage, 90°C	−0.12[e]

[a]This work.
[b]From [19].
[c]From [20].
[d]From [38].
[e]From [41].

and at pH 3.5, respectively [20]. The latter value refers to the pH-independent reaction. In contrast to moderately negative values obtained with aryl phosphates over a wide pH range, values of −1.28 and −0.12 have been reported for the hydroxide- and hydronium ion-catalysed cleavage of uridine 3′-alkyl phosphates (**2**) [41].

3.2 | Comparison of the Reactivity of 2-Hydroxypropyl Phosphates With Uridine 3′-Alkyl and Aryl Phosphates in the Absence of Metal Catalysts

The reactivity of three different phosphodiesters, uridylyl-3′,5′-uridine (3′,5′-UpU, **1**) [39], 2-hydroxypropyl (2-chlorophenyl)phosphate (HPCP, **4b**) and uridine 3′-(2-chlorophenyl)phosphate (UPCP, **3b**,) [42] is compared in Figure 2. Even though the different β_{LG} values suggest that the cleavage reactions of 2-hydroxypropyl arylphosphates (**4**) and nucleoside 3′-alkyphosphates (**2**) are clearly different, the reactivity of 2-hydroxypropyl ester **4b** (HPCP) and a dinucleoside monophosphate 3′,5′-UpU (**1**) are surprisingly similar. In contrast to this, the reactivity of a nucleoside 3-arylphosphate UPCP (**3b**) is clearly different. Under neutral and alkaline conditions, UPCP is 10^4 times more reactive than HPCP or 3′,5′-UpU are. Furthermore, a second-order dependence on hydronium ion concentration has not been observed with UPCP [42]. Despite clear differences in reactivity under neutral and alkaline conditions, at a low pH, UPCP reacts approximately at the same rate as 3′,5′-UpU and HPCP.

The three compounds, 3′,5′-UpU (**1**), UPCP (**3b**) and HPCP (**4b**) compared in Figure 2 form two pairs of substrates: one with the same nucleophile (2′-OH of uridine), but clearly different leaving groups, and another one with the same good 2-chlorophenol leaving group, but with different nucleophiles. The three compounds can be seen also as three different combinations of good and poor leaving groups and good and poor intramolecular nucleophiles. As the results in Figure 2 show, the good nucleophile–poor leaving group (3′,5′-UpU, **1**) and poor nucleophile–good leaving group (HPCP, **4b**) combinations show almost similar reactivity; only around neutral pH, HPCP is up to 10 times more reactive than 3′,5′-UpU is. The good nucleophile–good leaving group combination, as in UPCP (**3b**), results in a much more efficient reaction under slightly acidic, neutral and alkaline conditions, but under acidic conditions, the reactivity of the three substrates seems to approach the same value.

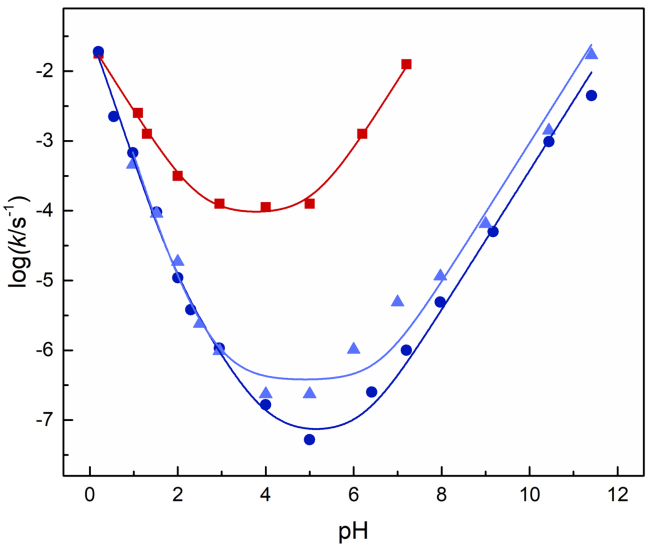


FIGURE 2 | pH-rate profiles for the cleavage of UPCP [42] (**3b**; red squares), HPCP (**4b**; light blue triangles) and 3′,5′-UpU [39] (**1**; navy blue circles).

The differences and similarities in the reactivity can be understood by evaluating the effects of the properties of different nucleophiles and leaving groups. The reactivity difference between HPCP (**4b**) and UPCP (**3b**), under conditions where base-catalysed reaction prevails, is the simplest case. The reaction of the uridine 3′-aryl phosphates **3**, such as UPCP, has been shown to be a concerted process [19], whereas in the case of 2-hydroxypropyl phosphates **4**, the nucleophilic attack is more clearly the rate-limiting step. The similarity of β_{LG} values, hence, most probably refers to the effect of the electron withdrawal by the leaving group on the efficiency of the nucleophilic attack. As the leaving group in HPCP (**4b**) and UPCP (**3b**) is the same, the reactivity difference can be attributed to the properties of the nucleophile.

According to the analysis by Lu et al. [23], the pK_a value of the 2-OH of a hydroxypropyl nucleoside phosphodiester is one pK_a unit higher than that of a 2′-OH in a ribonucleoside phosphodiester. Furthermore, the more rigid structure of the ribonucleoside moiety positions the nucleophile for the nucleophilic attack on the phosphate [16, 17], whereas the flexible structure of the 2-hydroxypropyl is less favourable for the nucleophilic attack. The difference in pK_a values shows that at a given pH, the proportion of the 2′-oxyanion nucleophile in UPCP (**3b**) is 10 times higher than that of the 2-oxyanion in HPCP (**4b**), and hence, the $10^{3.8}$-fold reactivity difference observed under neutral and alkaline conditions consists of two effects that disfavour the nucleophilic attack by 2-OH of HPCP (**4b**) in comparison 2′-OH of ribose in UPCP (**3b**): a 10-fold difference resulting from a lower concentration of the oxyanion nucleophile and an approximately $10^{2.8}$-fold difference that can be attributed to the less favourable orientation the nucleophile. It is to be noted that this analysis is based on two pairs of substrates with different nucleophiles and a similar good aryloxy-leaving group, and hence, the result may not be directly applicable in comparison between model compounds, such as HPNP (**4a**) and phosphodiester bonds in RNA.

The comparison between 3′,5′-UpU (**1**) and UPCP (**3b**) is less straightforward. Even though the attacking nucleophile is the same, and the substrates differ only in the structure of the leaving group, the reactivity difference cannot be attributed to the acidity of the leaving groups only. The β_{LG} values reported for the alkaline cleavage of uridine 3′-aryl (**3**) and alkylphosphates (**2**) are significantly different, −0.59 [19] and −1.10 [41], respectively. The effect of the acidity of the alkyl leaving groups has been taken as an indication of a reaction with a marginally stable phosphorane intermediate, where the departure of the alkoxy anion leaving group is the rate limiting step of the reaction [41]. In contrast to this, as discussed above, the more modest, but

TABLE 2 | The effect of the reaction conditions on catalytic activity of 10 mM CuTerPy and CuBiPy on the cleavage of HPNP (**4a**) and UPNP (**3a**).

Substrate/conditions	k (CuTerPy)/s^{-1}	k (CuBiPy)/s^{-1}	k (CuBiPy)/k (CuTerPy)
HPNP, pH 6, 90°C	$(3.84 \pm 0.11) \cdot 10^{-4}$ (60)	$(3.7 \pm 0.2) \cdot 10^{-3}$ (585)	9.8
HPNP, pH 7, 50°C	$(3.13 \pm 0.07) \cdot 10^{-5}$ (50)	$(1.18 \pm 0.01) \cdot 10^{-4}$ (190)	3.8
HPNP, pH 8, 25°C	$(2.87 \pm 0.08) \cdot 10^{-5}$ (54)	$(1.90 \pm 0.07) \cdot 10^{-5}$ (36)	0.66
UPNP, pH 6 25°C	$(8.1 \pm 0.2) \cdot 10^{-4}$ (42)	$(2.3 \pm 0.2) \cdot 10^{-3}$ (120)	2.8
UPNP, pH 7 25°C	$(8.5 \pm 0.5) \cdot 10^{-3}$ (42)	$(4.9 \pm 0.1) \cdot 10^{-3}$ (24)	0.57
UPNP, pH 8 2°C–3°C	$(2.5 \pm 0.2) \cdot 10^{-3}$ (14)	$(1.1 \pm 0.2) \cdot 10^{-3}$ (5.7)	0.48

Note: The values in the parenthesis are rate constants relative to those of uncatalysed reactions under the same conditions.

still significant β_{LG} values determined for the cleavage uridine 3′-arylphosphates **3** reflect the effect of the leaving group on the nucleophilic attack. When we compare the reactivity of 3′,5′-UpU (**1**) and UPCP (**3b**), we more or less compare the departure of the leaving group of one substrate and the nucleophilic attack in the case of the other substrate.

In the case of HPCP (**4b**), the nucleophilic attack more clearly determines the rate of the reaction, and the situation is less ambiguous. The equal reactivity of HPCP (**4b**) and 3′,5′-UpU (**1**) under conditions where the base-catalysed reaction predominates, shows that the energy barriers for the nucleophilic attack in the reaction of HPCP, and for the departure leaving group in the reaction of 3′,5′-UpU are approximately as high. The modest reactivity difference between HPCP and 3′,5′-UpU between pH 4 and 8 most probably reflects the fact that the more acidic phenolic leaving group is better able to depart as an oxyanion, whereas the departure of alkyl leaving group requires protonation of the leaving group oxygen. Below pH 4, where both substrates react by acid-catalysed mechanism, the reactivity of HPCP and 3′,5′-UpU is practically the same.

First- and second-order dependence on $[H^+]$ observed under acidic conditions refer to formation of neutral and cationic phosphates, which are more susceptible for a nucleophilic attack than the anionic one [39]. Protonation also facilitates the departure of the leaving group by allowing a proton transfer from the phosphorane intermediate to the leaving group oxygen. With UPCP (**3b**), the proton transfer may not be necessary [42], but the leaving group may depart also as an aryloxy anion. Detailed studies with 3′,5′-UpU (**1**) have shown that under acidic conditions, the transesterification reaction is a two-step process with a phosphorane intermediate that is sufficiently stable to pseudorotate [39]. Consequently, two transesterification reactions, isomerization and cleavage, are observed as shown in Scheme 1. The energy profile for the formation and the decomposition of the phosphorane intermediate in the reaction of 3′,5′-UpU under acidic conditions most probably is fairly symmetric [39]. Considering this, the similar reactivity of 3′,5′-UpU (**1**) and HPCP (**4b**) means that the energy barrier for the nucleophilic attack 2-OH of the hydroxypropyl moiety in **4b** on a protonated phosphate cannot be significantly higher than those of either step in the reaction of 3′,5′-UpU. This shows that the disadvantage brought about by the flexibility of the nucleophile can be overcome by the protonation of the phosphate.

3.3 | Comparison of CuTerPy and CuBiPy as Catalysts of the Cleavage of RNA Model Compounds

Reactions of different types of RNA model compounds were studied also in the presence of CuTerPy and CuBiPy complexes. First, reactions of two different RNA models, uridine 3′-*p*-nitrophenylphosphate (UPNP, **3a**) and 2-hydroxypropyl 1- *p*-nitrophenylphosphate (HPNP, **4a**), with the same good 4-nitrophenol leaving group were studied at three different pH as a function of the catalyst concentration. The conditions were chosen to allow a convenient analysis of each substrate, and to be able to evaluate the effect of dimerization of CuBiPy, which according to the analysis by Garribba et al. [30] is negligible at pH 6, and complete at pH 8. The comparison of results obtained with 10 mM catalysts is shown in Table 2; the whole set of results can be found in Supporting information S1.

As the reactivity of the substrates is clearly different, reactions were carried out at different temperatures, and consequently, the stability constants of the catalyst-substrate complexes, as well as the pK_a values of the Cu^{2+}-bound aquo ligands are different. Results obtained with two different substrates cannot, hence, be directly compared, whereas experiments with the same substrate and two different complexes under the same conditions reveal a difference in the catalytic activity of the complexes. It is to be noted that terms catalyst and catalytic activity are used throughout the text for convenience, even though the complexes act most probably as stoichiometric reagents.

The results collected in Table 2 show that at pH 6, CuBiPy is more efficient than CuTerPy as the catalyst with both UPNP (**3a**) and HPNP (**4a**) as substrates. The difference is more apparent with HPNP (**4a**) at 90°C than with the nucleoside derivative UPNP (**3a**) at 25°C. At pH 7 and at 50°C, CuBiPy is still more efficient as a catalyst in the reaction of HPNP (**4a**), but with UPNP (**3a**) as a substrate, the rate enhancement by CuTerPy at 25°C is slightly larger. At pH 8, the trend is apparent: CuTerPy is the more efficient catalyst with both HPNP and UPNP as substrates.

The difference between CuTerPy and CuBiPy, and the effect of different conditions on the efficiency of CuBiPy as a catalyst can be, at least partly, attributed to the formation of the hydroxobridged CuBiPy dimer [30], and to the lower pK_a of the aquo ligand of CuBiPy. pK_a values of 6.6 and 8.1 have been reported for CuBiPy and CuTerPy, respectively [27]. The

data obtained in the present study (collected in Table S1 in Supporting information S1) do not unambiguously confirm the formation of CuBiPy dimers under the experimental conditions of the present work. However, it is clear that under conditions where the dimeric form of CuBiPy, according to the previous analysis prevails [30], the catalysis by CuBiPy in comparison with CuTerPy becomes less efficient. It would also seem that the effect is less pronounced with UPNP (**3a**) as the substrate, but as the experimental conditions are different, no firm conclusions can be drawn.

3.4 | The Effect of the Nucleophile on the CuTerPy and CuBiPy Catalysed Reactions

In contrast to the results discussed above, the data in Table 3 clearly show that different substrates show a different preference for the catalyst. Entries 1–3 show a comparison between three different substrates, UPNP (**3a**), HPNP (**4a**) and bis-(*p*-nitrophenyl)phosphate (BPNP, **5**) with the same leaving group, but with a different nucleophile. This comparison shows that although CuTerPy is slightly more efficient catalyst in cleavage of UPNP (**3a**) with the 2′-OH of ribose moiety as a good intramolecular nucleophile, CuBiPy is more efficient in the reaction of HPNP (**4a**) with a poorer intramolecular nucleophile. Furthermore, an even larger difference in favour of CuBiPy is observed with BPNP (**5**) in a reaction which involves an external HO^- ion as a nucleophile. Entries 4–6 show results obtained with another series of substrates: three different 2-hydroxypropylphosphates HPNP (**4a**), HPCP (**4b**) and HPHP (**4c**), that is, compounds with the same nucleophile, but with different leaving groups. Consistent with results shown in Table 2, CuBiPy is a more efficient catalyst for the reaction of HPNP (**4a**). However, as the leaving group becomes poorer, as with HPCP (**4b**) and HPHP (**4c**), CuTerPy is becomes the more efficient catalyst.

It seems, hence, that the interplay of the nucleophile and the leaving group determines which Cu^{2+} complexes is the more efficient catalyst for the cleavage of a given phosphodiester. A poor nucleophile together with a good leaving group, as in the case of BPNP (**5**) or HPNP (**4a**), favours the catalysis by CuBiPy, but when the 'poorness' of the nucleophile in relation to 'goodness' of the leaving group decreases, catalysis by CuTerPy becomes more efficient. With HPNP (**4a**), HPCP (**4b**) and HPHP (**4c**) the situation is not as clear. As discussed in Section 3.1, the nucleophilic attack is the rate-limiting step of the cleavage of 2-hydroxypropyl phosphates (**4**), but even then, the electronegativity of the leaving group affects the reactivity. It is clear, however, that CuTerPy becomes the more favoured catalyst as the leaving group becomes poorer while the nucleophile remains the same. Similar observation can be seen in previous results with a series of uridine 3′-arylphosphates [18]. When the acidity of the leaving group decreases, the preference for CuTerPy as a catalyst becomes more apparent. In the reaction of uridine 3′-phenylphosphate (**3**; X = H), CuTerPy is 4.4 times more efficient as a catalyst than CuBiPy at pH 6.5 and 25°C [18].

The interplay and its effect become more apparent when the rate enhancements observed with different substrates are compared. As is shown by the results in Tables 2 and 3, the rate enhancement by CuTerPy and CuBiPy observed with 2-hydroxypropyl phosphates **4a–c** and UPNP (**3a**) as substrates is fairly modest. Generally, the rate enhancements are larger with HPNP (**4a**) as a substrate than with UPNP (**3a**). Clearly larger rate-enhancing effects were observed with BNPP (**5**) as a substrate: at pH 7.0 and 50°C, 2-mM CuTerPy and CuBiPy enhance the reaction of BPNP 302 and 8200-fold, respectively. Under these conditions, CuBiPy most probably is at least partially dimerized. At a lower pH, the proportion of the catalytically more efficient monomer is higher, and the rate enhancement would most probably be even more significant. Consistent with this, a 585-fold rate enhancement of the cleavage of HPNP (**4a**) by 10-mM CuBiPy was

TABLE 3 | Comparison of CuBiPy and CuTerPy as catalysts of the cleavage of different phosphodiesters.

Entry	Substrate	Conditions	*c* (Cu)/mM	*k* (CuBiPy)/s^{-1}	*k* (CuTerPy)/s^{-1}	*k* (CuBiPy)/*k* (CuTerPy)
1	BPNP (**5**)	pH 7, 50°C	2	$(3.4 \pm 0.3) \cdot 10^{-5}$ (8200)[a]	$(12.4 \pm 0.3) \cdot 10^{-7}$ (302)[a]	27
2	HPNP (**4a**)	pH 7, 50°C	2	$(44.8 \pm 0.5) \cdot 10^{-6}$ (72)[b]	$(6.57 \pm 0.09) \cdot 10^{-6}$ (11)[b]	6.8
3	UPNP (**3a**)	pH 7, 50°C	2	$(1.37 \pm 0.08) \cdot 10^{-2}$ (6)[c]	$(2.91 \pm 0.08) \cdot 10^{-2}$ (12)[c]	0.5
4	HPNP (**4a**)	pH 6.7, 90°C	5	$(3.01 \pm 0.03) \cdot 10^{-3}$ (358)[d]	$(1.04 \pm 0.02) \cdot 10^{-3}$ (123)[d]	2.9
5	HPCP (**4b**)	pH 6.7, 90°C	5	$(1.05 \pm 0.03) \cdot 10^{-4}$ (46)[d]	$(1.89 \pm 0.06) \cdot 10^{-4}$ (82)[d]	0.47
6	HPHP (**4c**)	pH 6.7, 90°C	5	$(7.9 \pm 0.6) \cdot 10^{-6}$ (7)[d]	$(4.5 \pm 0.1) \cdot 10^{-5}$ (46)[d]	0.15

[a]Rate constant $4.2 \cdot 10^{-9}\,s^{-1}$ for uncatalysed reaction of BPNP has been estimated from data in [31].
[b]Rate constant $(6.2 \pm 0.4) \cdot 10^{-7}$ for uncatalysed reaction of HPNP has been determined in the present work.
[c]Rate constant $4.7 \cdot 10^{-4}\,s^{-1}$ for uncatalysed reaction of UPNP is from [18].
[d]Rate constants $8.4 \cdot 10^{-6}\,s^{-1}$, $2.43 \cdot 10^{-6}\,s^{-1}$ and $9.8 \cdot 10^{-7}\,s^{-1}$ for uncatalysed reaction of HPNP, HPCP and HPHP has been obtained by interpolation from data in Figure 1.

TABLE 4 | β_{LG} values for the cleavage of different RNA models in the presence CuTerPy and CuBiPy catalysts.

Substrates	β_{LG} (uncat.)	β_{LG} (CuTerPy)	β_{LG} (CuBiPy)
2-Hydroxypropyl phosphates HPNP (4a), HPCP (4b) and HPHP (4c)[a]	−0.42 ± 0.01	−0.57 ± 0.01	−0.98 ± 0.05
Uridine 3′-arylphosphates 3[b]	−0.7 ± 0.1	−0.7 ± 0.1	−0.9 ± 0.1
Uridine 3′-alkylphosphates 2[c]	−0.92 ± 0.03	−0.48 ± 0.05	−0.43 ± 0.04

[a]This work, pH 6.7, 90°C.
[b]Calculated on the basis of data in [18], pH 6.6, 25°C.
[c]Korhonen et al. [18], pH 6.6, 90°C.

observed at pH 6.0 and 90°C (Table 2), in contrast to 72-fold rate enhancement at pH 7 and 50°C (Table 3).

The observation of a higher catalytic activity with more unreactive substrates is not limited to CuTerPy and CuBiPy. In addition to small-molecular weight catalysts, similar results have been reported for example with cyclodextrin based catalysts [43, 44], a dimeric catalyst based on Zn^{2+}-triazanonane complexes [45, 46] and peptide-based RNase and DNase mimics [10]. Czescik et al. [13] have also reported that a gold nanoparticle nanozyme catalyses the hydrolysis of HPNP (**4a**) clearly more efficiently than that of UPNP (**3a**). However, there are also examples of catalysts that show no particular preference for BPNP (**5**) over HPNP (**4a**) as a substrate [46].

3.5 | Mechanistic Implications

The conclusion of the results discussed above is that the largest rate enhancement by CuTerPy and CuBiPy is observed when the nucleophilic attack clearly determines the rate of the uncatalysed cleavage. The catalytic activity of the complexes decreases as the nucleophile becomes better while the leaving group remains the same. Correspondingly, although more modest, the effect is seen when the nucleophile remains the same, either 2-OH of 2-hydroxypropyl phosphates (**4**) or 2′-OH of uridine 3′-arylphosphates (**3**), and the leaving group becomes poorer: the catalytic activity of CuTerPy and CuBiPy decreases when the acidity of the phenolic leaving group decreases. Furthermore, our previous results with uridine 3′-alkyl phosphates (**2**) show that the different types of phosphodiesters form a mirroring system [18]. With these substrates, the departure of the poor leaving group is the rate-limiting step of the uncatalysed cleavage. The results obtained in the presence of CuTerPy and CuBiPy show that their catalytic activity increases as the leaving group becomes poorer and more clearly limits the rate of the reaction. While the cleavage of uridine 3′-trichloroethyl phosphate (pK_a of the leaving group 12.2 [47]) was enhanced by factors of 11 and 61 by 10 mM CuBiPy and CuTerPy, respectively, at pH 6.6.and 90°C, the corresponding values for an ethoxyethyl phosphate (pK_a 14.8 [47]) were 291 and 2164, and for a neo-pentyl phosphate (pK_a 17.3 [47]) 1900 and 13000 [18].

Although the catalytic activities of both CuTerPy and CuBiPy change in the same direction when the properties of the nucleophile or the leaving group change, it is clear that as catalysts they favour different types of substrates. CuBiPy is a better catalyst when the nucleophilic attack determines the rate of the reaction, whereas CuTerPy becomes the better catalyst when the effect of the leaving group increases. This suggests that the complexes utilize different catalysis mechanisms in the cleavage of different phosphodiesters. This is shown more quantitatively by β_{LG} values collected in Table 4. Although the values obtained for the uncatalysed and CuTerPy promoted cleavage of 2-hydroxypropyl phosphates **4a–c** are fairly modest, the value obtained in the presence of CuBiPy is clearly more negative. Less significant effect is seen in the values calculated for the cleavage of uridine 3′-arylphosphates **3** on basis of results reported in Korhonen et al. [18]. In contrast to this, the β_{LG} values obtained for the cleavage of uridine 3′-alkylphosphates **2** in the presence of CuTerPy and CuBiPy are approximately the same and clearly less negative than the value for the uncatalysed reaction under the same conditions.

The most straightforward way to understand these results is to suggest that if the β_{LG} values for the Cu^{2+}-complex catalysed and uncatalysed reactions are the same, the complex does not affect the protonation state of the substrate. Alternatively, two opposite effects cancel each other. Different values, in contrast, show that the catalyst interacts either with the nucleophile, or with the leaving group. The results collected in Table 4 suggest that CuTerPy and CuBiPy complexes utilize different catalysis mechanisms in the cleavage of 2-hydroxypropyl arylphosphates **4**, and possibly also with uridine 3′-arylphosphates **3**. Furthermore, the catalysis mechanism utilized by the complexes with nucleoside 3′-alkylphosphates **2** as substrates are probably different from those utilized with arylphosphate substrates **3** and **4**.

4 | Conclusions

The results presented in this paper show that even though the rate-limiting steps are different, two different RNA model compounds, one with a good nucleophile and a poor leaving group (3′,5′-UpU; **1**) and another one with a poor nucleophile and good leaving group (HPCP; **4b**), are cleaved approximately at the same rate in the absence of metal ion catalysts. In contrast to this, the alkaline cleavage of a substrate with a good nucleophile and a good leaving group (UPCP; **3b**) is approximately 10^4 times more efficient. The comparison between a nucleoside-based (**3**) and 2-hydroxypropyl arylphosphates (**4**) shows that an approximately 10^3-fold difference arises from the unfavourable flexibility of the 2-hydroxypropyl nucleophile, the rest being a result of a difference in deprotonation of the nucleophile. Under acidic conditions, the reactivity of all three types of substrates studied approaches the same value. Apparently, protonation of the

phosphate brings the energy barrier for nucleophilic attack by a poor 2-OH nucleophile in 2-hydroxypropyl phosphates at the same level with that of 2′-OH of ribose.

The results obtained in the presence of CuTerPy and CuBiPy reflect the properties of the nucleophile and the leaving group: the more clearly either the nucleophilic attack or the departure of the leaving group determines the rate of the uncatalysed reaction, the larger is the rate enhancement by metal ion catalysts. The results obtained show also that CuBiPy is more efficient as a catalyst in cases where the nucleophile is poor and the leaving group is good. CuTerPy is more efficient with substrates with a good nucleophile and a poor leaving group. Between two extremes, there is a continuum where the relative importance of the nucleophilic attack and the departure of the leaving group gradually changes. The comparison of β_{LG} values suggests that CuTerPy and CuBiPy utilize different catalytic strategies and that the mechanism of the catalysis depends also on the structure of the substrate. In any case, it is obvious that the most efficient catalysis of the cleavage of an RNA phosphodiester bond would be achieved multifunctional catalyst consisting of different catalytic entities to enhance both steps of the reaction.

Author Contributions

Jasmin I. Koski: investigation. **Emilia Poijärvi:** investigation. **Anne Tulisalo:** investigation. **Heidi Korhonen:** supervision, data curation. **Satu Mikkola:** investigation, writing – original draft, writing – review and editing, project administration, supervision, conceptualization, data curation.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.