CHAPTER 1

DESIGN, SYNTHESIS, AND APPLICATIONS OF NUCLEOSIDE PHOSPHATE AND PHOSPHONATE PRODRUGS

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1.1. INTRODUCTION

For decades, numerous nucleoside analogs have been developed as drugs or drug candidates [1, 2] for the treatment of various cancers and infections by different viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), and human cytomegalovirus (HCMV). In cells, nucleoside analog molecules are converted to the corresponding phosphates by nucleoside or nucleotide kinases [3, 4] and then are incorporated into viral or tumor DNA/RNA where they serve as chain terminators. For example, the anti-HIV nucleoside drug azvudine (FNC) [4–6] is further phosphorylated *in vivo* to the active nucleoside triphosphate (FNC-TP, Figure 1.1), which inhibits the viral replication. However, the structural differences between the nucleoside analogs and the natural nucleosides may affect the phosphorylation processes, thus decreasing the pharmacological activity of the compounds [7, 8]. Due to their poor chemical stability and high polarity, the corresponding phosphates themselves cannot be used directly as drugs

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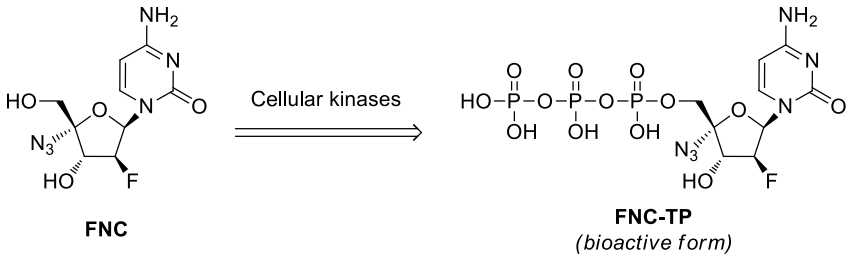


Figure 1.1 Formation *in vivo* of the active triphosphate (TP) of FNC.

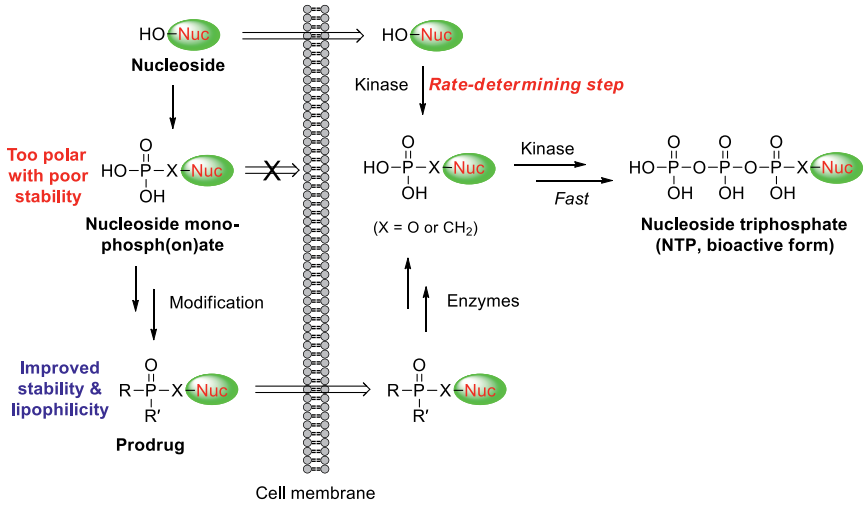


Figure 1.2 Design and mechanism of action of nucleoside phosph(on)ate prodrugs.

(Figure 1.2). To address these issues, diverse prodrugs of nucleoside (nucleotide) phosph(on)ate analogs have been designed and synthesized in drug discovery [3, 9–14].

Introduction of phosph(on)ate esters as prodrugs (Figure 1.2) has emerged as a very useful tool in the design and discovery of nucleoside and nucleotide analog drugs for the treatment of cancers and viral infectious diseases. The features of a prodrug may include (1) improved chemical stability; (2) increased lipophilicity for better bioavailability; (3) oral availability (the parent compound may often be administered only by injection); and (4) an improved therapeutic effect with reduced toxicity *via* targeted drug delivery. In recent years, considerable effort has been devoted to the design, synthesis, and biological evaluation of nucleoside phosph(on)ate prodrugs and more than twenty drug candidates have entered clinical development. Of these, several prodrugs have received FDA approval for clinical use. These include

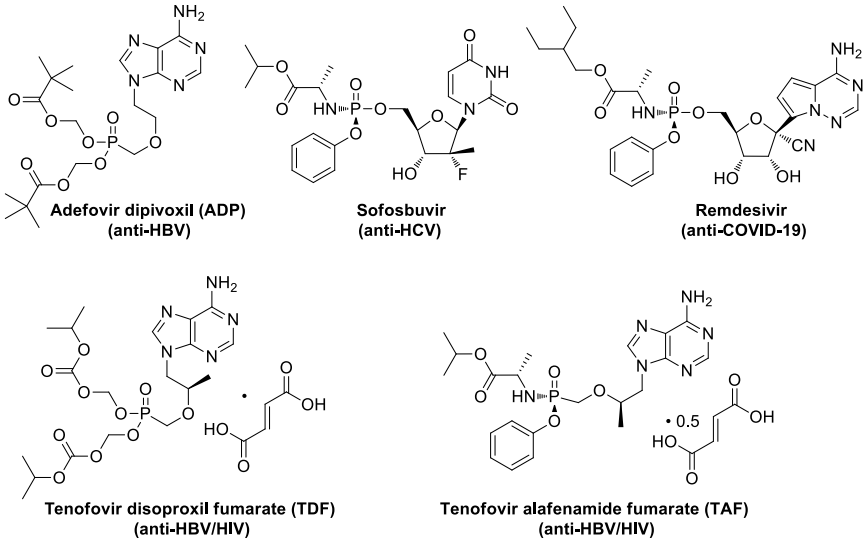


Figure 1.3 Nucleoside phosph(on)ate prodrugs in clinical use.

ADP, TDF, sofosbuvir, TAF, and remdesivir (Figure 1.3). In this chapter, four main classes of nucleoside phosph(on)ate prodrugs and prodrug candidates are discussed: carbonyloxymethyl diester prodrugs, alkoxyalkyl monoester prodrugs, cyclic 1-aryl-1,3-propanyl ester (HepDirect) prodrugs, and phosphoramidate/phosphonamidate prodrugs.

1.2. NUCLEOSIDE PHOSPH(ON)ATE PRODRUGS

1.2.1. Carbonyloxymethyl Diester Prodrugs

Two nucleotide prodrugs developed *via* this strategy, ADP (trade names: Preveon and Hepsera) and TDF (trade name: Viread), have been approved by FDA for the treatment of viral infectious diseases. Both the acyclic nucleoside phosphonate drugs have a carbonyloxymethyl diester moiety. Specifically, ADP contains a bis(pivaloyloxymethyl) (POM) substructure and TDF bears a bis(isopropyloxymethyl carbonate) group (POC). These carbonyloxymethyl (e.g., POM) and alkyloxycarbonyloxyalkyl (e.g., POC) diester units can increase the oral bioavailability and overall systemic exposure when compared to the parent nucleotide molecules. Activation of this carbonyloxymethyl ester prodrug involves the esterase-catalyzed cleavage of the first carbonate ester group followed by chemical degradation to form an unstable POM- or POC-monoester (**2**). The monoester intermediate then undergoes a second degradation cycle to afford the expected nucleoside monophosph(on)ate (**1**)

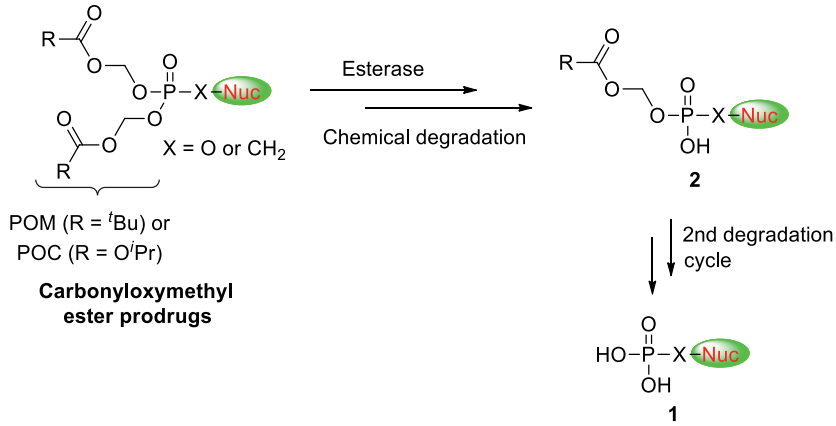


Figure 1.4 Activation of carbonyloxymethyl ester prodrugs.

(Figure 1.4). In addition to the above two marketed nucleotide prodrugs, besifovir (**LB80380**) is a bis(POM)-type prodrug candidate of an acyclic purine nucleotide analog and has entered clinical development for the treatment of HBV infection.

1.2.1.1. Adefovir Dipivoxil Adefovir (ADV), also known as 9-(2-phosphonomethoxyethyl)adenine (PMEA), is an acyclic nucleotide analog of adenosine monophosphate. ADV was first reported by De Clercq *et al*. [15] as having potent antiviral activity against HIV and other retroviruses [16, 17]. To increase the lipophilicity and intestinal permeability of the parent compound (ADV) for oral administration, various prodrugs of adefovir have been designed. However, the previously designed prodrugs with simple alkyl diester or amide groups failed, probably due to their inefficient degradation to adefovir *in vivo* [18], and the monoesters showed poor oral bioavailability because of the unmasked negative charge. Eventually, ADP (bis-POM PMEA, Figure 1.5) designed by masking the phosphonic acid

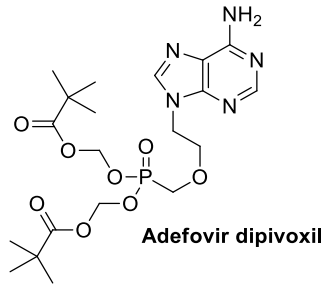


Figure 1.5 Structure of adefovir dipivoxil.

moiety with two POM groups was identified and was found to have favorable lipophilicity and intestinal permeability. It can be conveniently metabolized to the parent nucleotide adefovir *in vivo*, leading to high oral bioavailability. ADP was initially developed for the treatment of HIV infections (AIDS) [19, 20], but failed due to its toxicity. Further development demonstrated that this ADV prodrug can significantly reduce the HBV viral load at a nontoxic dose [21, 22]. ADP (Preveon and Hepsera) received FDA approval for the treatment of chronic HBV infection *via* oral administration as a reverse transcriptase (RT) inhibitor.

1.2.1.2. Tenofovir Disoproxil Fumarate Tenofovir, (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA), was designed by incorporation of a methyl group into the side chain of adefovir. As a nucleotide analog reverse transcriptase inhibitor (nRTI), PMPA exhibits good inhibitory activity against HIV and other retroviruses [23]. However, like other nucleotide analogs, the parent nucleotide analog suffers from poor oral bioavailability. To improve the oral delivery and also avoid the side effects [24] from pivalic acid derived from ADP, new prodrugs were designed and evaluated. Among these, the bis(isopropyloxycarbonyloxym ethyl) ester (bis-POC) prodrug (tenofovir disoproxil) displayed favorable solubility, stability, and improved oral bioavailability compared to the parent nucleotide and thus was selected for clinical development [25, 26]. TDF (Figure 1.6) has been approved by the FDA for the treatment of HIV/AIDS as well as chronic hepatitis B under the trade name Viread.

1.2.1.3. Besifovir Besifovir (**LB80380**, Figure 1.7) is a bis(POM) phosphonate prodrug of a 2-aminopurine nucleotide analog and is in clinical trials for the treatment of HBV infection [27]. **LB80380** displayed potent anti-HBV activity against wild-type and drug-resistant strains [28]. In the liver, the prodrug will be rapidly degraded to release the parent nucleotide, which is then oxidized at the C6-position of the purine moiety followed by phosphorylation to form the bioactive metabolites that serve as inhibitors of HBV DNA replication [27, 29].

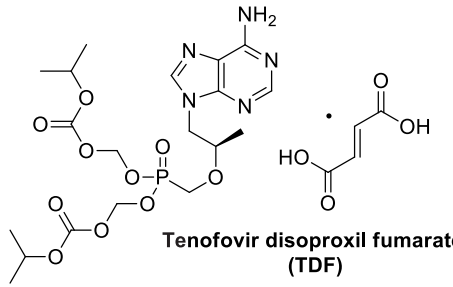


Figure 1.6 Structure of tenofovir disoproxil fumarate (TDF).

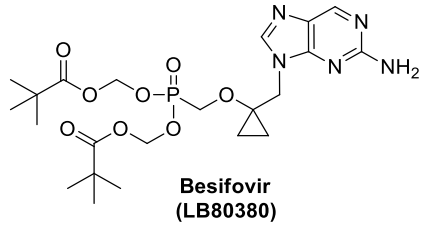


Figure 1.7 Structure of besifovir (**LB80380**).

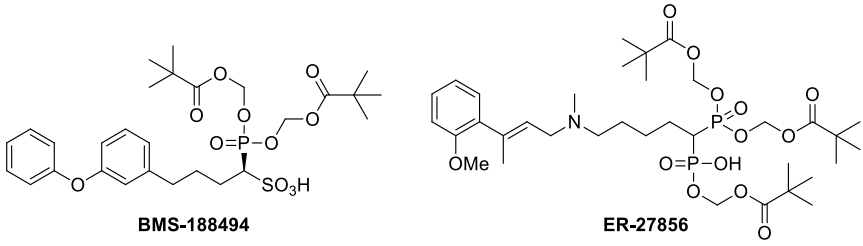


Figure 1.8 Structure of non-nucleoside prodrugs **BMS-188494** and **ER-27856**.

Application of the carbonyloxymethyl ester prodrug strategy to non-nucleoside compounds led to the discovery of **BMS-188494**, a bis(POM)-substituted squalene synthase inhibitor (Figure 1.8), which is in clinical trials for the treatment of hypercholesterolemia [30]. Another squalene synthase inhibitor bearing three POM groups (**ER-27856**) (Figure 1.8) was demonstrated to have a cholesterol-lowering effect [31] and inhibitory activity on the growth of *Trypanosoma cruzi* [32].

1.2.2. Alkoxyalkyl Monoester Prodrugs

In most of the phosph(on)ate prodrugs, both oxygens of the phosph(on)ate groups are fully masked, but in this prodrug approach, only one of them is masked and the other remains as a free OH group. The concept underlying the alkoxyalkyl monoester prodrug design is to mimic the structural features of the natural lysophosphatidylcholine (LPC). Further structural optimization of the masking groups led to hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) derivatives, two important prodrug moieties. These prodrugs are orally available, and they may be delivered into the targeted tissues through the same uptake pathway in the small intestine as LPC. After reaching the desired tissue, the prodrug is cleaved by specific cellular enzymes such as phospholipase C to release the nucleoside monophosph(on)ate (**1**) (Figure 1.9). The drug candidates brincidofovir (**CMX-001**), **CMX-157**, and fozivudine tidoxil belong to the alkoxyalkyl monoester prodrug class.

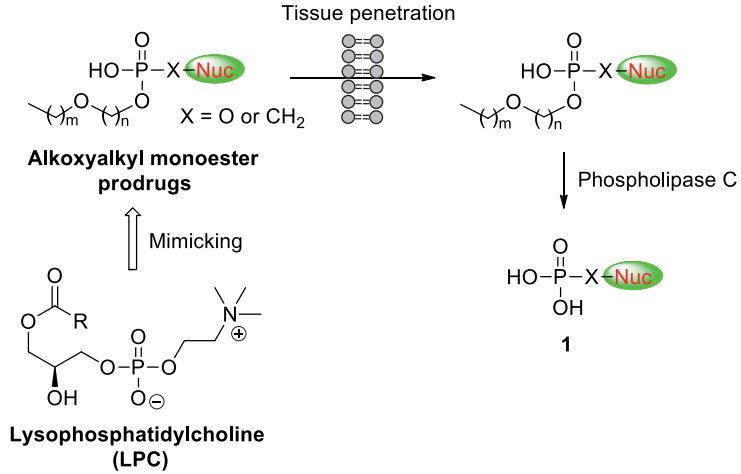


Figure 1.9 Design and activation of alkoxyalkyl monoester prodrugs.

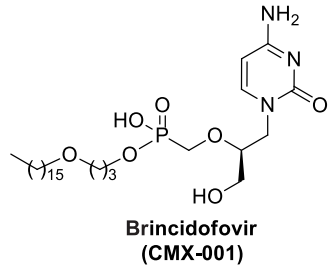


Figure 1.10 Structure of brincidofovir (**CMX-001**).

1.2.2.1. Brincidofovir Cidofovir (CDV) is an FDA-approved injectable antiviral agent targeted to treat smallpox infections and complications caused by smallpox vaccines. To improve the lipophilicity of CDV and enhance the uptake of the orally administered drug in the small intestine, the prodrug brincidofovir (**CMX-001**, Figure 1.10) was designed with a lipid HDP monoester group linked to the phosphonate moiety [33]. As expected, **CMX-001** is more lipophilic than the parent compound and could be absorbed by active transport in the small intestine, resulting in a more rapid accumulation in the target cell cytoplasm. After being degraded to the parent nucleotide (CDV), it is further phosphorylated by host cell nucleoside kinases into cidofovir diphosphates, which serve as viral DNA polymerase inhibitors. **CMX-001** displays broad-spectrum antiviral activity against adenoviruses, smallpox, cytomegalovirus (CMV), papillomavirus, polyomavirus, and orthopoxviruses [34]. As an orally available drug candidate, brincidofovir

has entered clinical development for the treatment of smallpox infections and complications related to the smallpox vaccine.

1.2.2.2. CMX-157 **CMX-157** (Figure 1.11) is a HDP monoester prodrug candidate of nucleotide analog tenofovir and is in clinical trials [35] as a potential treatment for HIV-1 and HBV infections. A lipophilic HDP group has been incorporated into this prodrug to mimic natural LPC for efficient drug delivery through the LPC uptake pathway and thus to improve oral availability, efficacy, and the toxicity profile of tenofovir [35, 36]. As expected, **CMX-157** displays > 300-fold better antiviral activity than the parent compound and is active against most of the HIV-1 virus subtypes [36].

1.2.2.3. Fozivudine Tidoxil Fozivudine tidoxil (Figure 1.12) is a prodrug of zidovudine (AZT) developed *via* the alkoxyalkyl monoester prodrug strategy with a similar thioether lipid moiety instead of the HDP group. Fozivudine tidoxil has progressed into phase II clinical trials for the treatment of patients with HIV infection.

1.2.3. Cyclic 1-Aryl-1,3-propanyl Ester (HepDirect) Prodrugs

A HepDirect prodrug approach [8, 10, 37, 38] was developed by Metabasis Therapeutics in the early 2000s. Prodrugs from this work contain cyclic 1-aryl-1,3-propanyl ester moieties, which possess high plasma and tissue stability. They can be metabolized by cytochrome P_{450} (CYP) enzymes that are

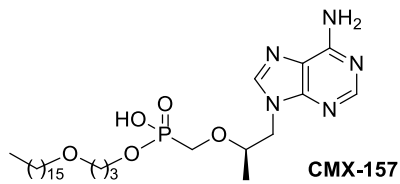


Figure 1.11 Structure of **CMX-157**.

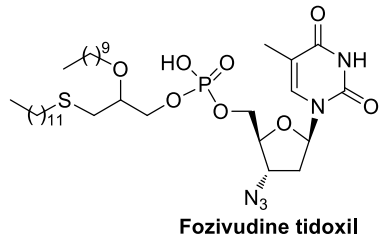


Figure 1.12 Structure of fozivudine tidoxil.

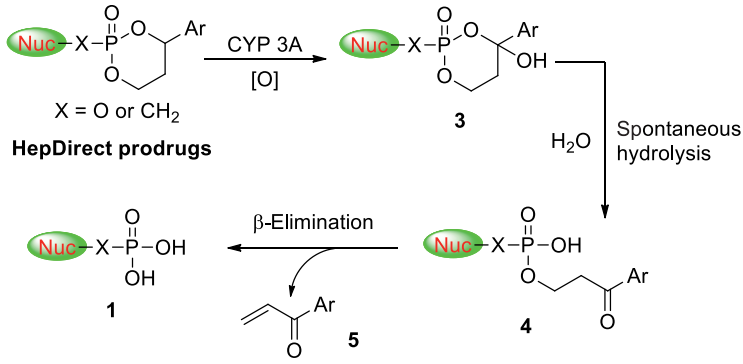


Figure 1.13 Activation of HepDirect prodrugs.

expressed predominantly in the liver to achieve liver-targeted drug delivery. The CYP-catalyzed degradation of the prodrugs results in oxidative hydroxylation at the benzylic position (**3**). Subsequently, ring-opening of the intermediate (**3**) followed by β-elimination liberates the expected nucleoside phosph(on)ate (**1**) (Figure 1.13). The resulting vinyl ketone (**5**) may further conjugate with glutathione to form less toxic metabolites. Nucleoside or nucleotide prodrug candidates developed *via* this strategy, which are in clinical trials, include pradefovir (**MB06866**) and **MB07133**.

1.2.3.1. Pradefovir Pradefovir (**MB06866**, Figure 1.14) is a HepDirect prodrug candidate of adefovir bearing a 3-chlorophenyl group. It was developed for the treatment of hepatitis B virus infection [38].

The previously marketed anti-HBV drug ADP is a carbonyloxymethyl diester prodrug of the acyclic nucleotide adefovir. ADP was originally developed as an antiviral agent against HIV, but failed due to side effects. Although it received FDA approval for the treatment of chronic hepatitis B, ADP is only used at submaximally efficacious doses on account of its renal toxicity. To improve the therapeutic index, cyclic 1-aryl-1,3-propanyl ester (HepDirect) prodrugs of the parent compound were designed and synthesized to deliver the bioactive

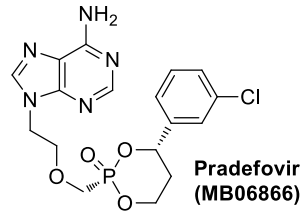


Figure 1.14 Structure of pradefovir (**MB06866**).

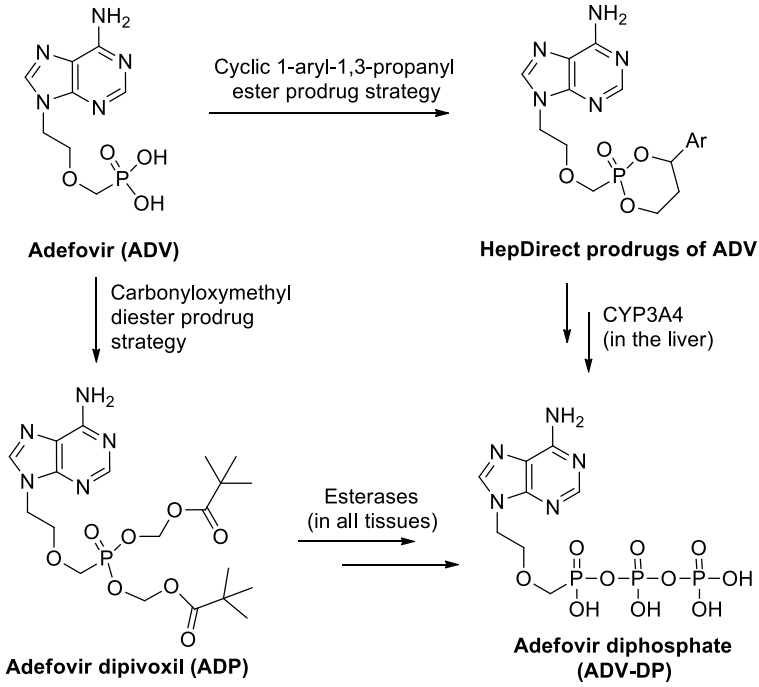


Figure 1.15 Design rationale of HepDirect prodrugs of adefovir.

adefovir diphosphate (ADP-DP) selectively to the liver (Figure 1.15) and thus reduce side effects by decreasing the exposure to the kidney [38].

Among the HepDirect prodrugs of ADV, the analog bearing a 3-chlorophenyl group displayed a high activation rate in microsomes, in primary hepatocytes, and orally administered in rats. Further studies suggested that the stereochemistry has different influences on the absorption and activation of the prodrug. Among the four stereoisomers, the racemic *cis*-isomers showed a high rate of activation but the racemic *trans*-isomers were not activated in human microsomes. The *cis*-isomers also gave a 12-fold improvement in the liver/kidney exposure ratio compared to ADP in rats *via* oral administration. The *cis*-(2*R*,4*S*) prodrug of the *cis*-diastereomers is the better-activated compound than the other *cis*-isomer. Evaluation of the *cis*-diastereomers led to the selection of the *cis*-(2*R*,4*S*) prodrug isomer in the form of the mesylate salt (**MB06866**) as the drug candidate for further development (Figure 1.16). Pradefovir (**MB06866**, previously known as remofovir) displayed favorable oral bioavailability in rats and dogs and has been advanced into clinical trials for the treatment of hepatitis B patients.

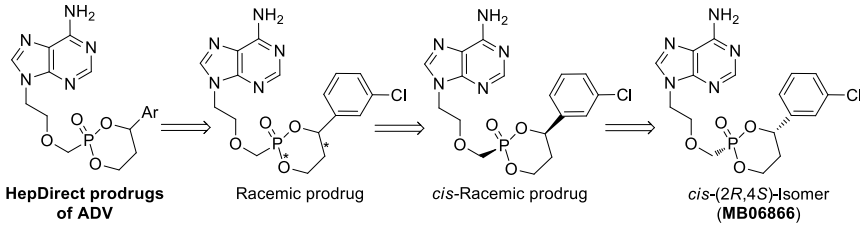
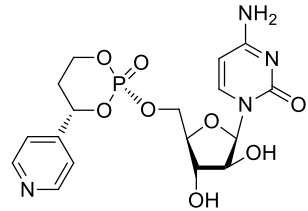


Figure 1.16 Lead optimization for the discovery of pradefovir (**MB06866**).

1.2.3.2. MB07133 Cytarabine is a pyrimidine nucleoside analog in clinical use for the treatment of acute myelocytic leukemia [39]. Like many nucleoside analogs, cytarabine has a limited phosphorylation rate forming the bioactive nucleoside triphosphate (NTP) *in vivo*, and the higher doses necessary to achieve the therapeutic NTP level may cause side effects, such as myelosuppression [40]. Application of the HepDirect prodrug strategy to this cytidine analog led to **MB07133** (Figure 1.17), a prodrug candidate to treat hepatocellular carcinoma (HCC) [40]. This prodrug with its 1-(4-pyridyl)-1,3-propanyl ester group exhibited profiles in activation, stability and solubility favorable for parenteral administration during the lead optimization. Compared to the parent nucleoside, **MB07133** can achieve much higher levels of the active cytarabine triphosphate in the liver than in the bone marrow and the plasma [40].

The HepDirect prodrug strategy was also involved in the discovery of non-nucleoside drug candidates. For example, **MB07811** is a liver-activated prodrug candidate of a phosphonic acid (PA) thyroid hormone receptor (TR) agonist and is used for the treatment of hyperlipidemia [41] (Figure 1.18). It displays potent lipid-lowering activity in a cholesterol-fed rat (CFR) model and good oral bioavailability ($F = 40\%$) in rats.



MB07133

Figure 1.17 Structure of **MB07133**.

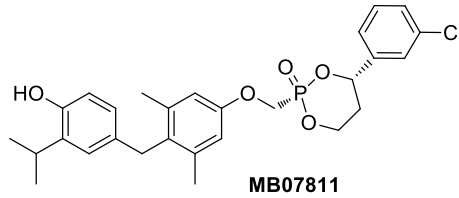


Figure 1.18 Structure of non-nucleoside prodrug **MB07811**.

1.2.4. Phosphoramidate and Phosphonamidate Prodrugs

In the early 1990s, McGuigan *et al*. developed the aryloxy phosphoramidate prodrug approach, also known as ProTide (PROdrug + nucleoTIDE) technology, to deliver nucleoside monophosphates into cells. Generally, in ProTide prodrugs, there is an amino acid alkyl ester and an aryloxy group connected to a phosphorus atom. During the development of this type of prodrug, a variety of related prodrugs were designed and synthesized [3], including di(halo)alkyl phosphates, (halo)alkyloxy phosphoramidates, phosphorodiamidates, diaryl phosphates, and alkyloxy phosphor-amidates (ProTides). Among these, the ProTide prodrugs are superior to other related drugs, as they not only can significantly increase or even reveal the biological activity of the parent nucleos(t)ides but also are more convenient to synthesize. To date, the ProTide technology has become a powerful tool for medicinal chemists in the discovery and development of new nucleos(t)ide prodrug candidates. This prodrug approach has led to three FDA-approved drugs, sofosbuvir (trade name: Sovaldi), TAF (trade name: Vemlidy), and remdesivir (trade name: Veklury), as well as a number of drug candidates for clinical development.

The mechanism of action of ProTide prodrugs [3, 42, 43] has been shown to be as illustrated in Figure 1.19. After entrance into cells, the metabolism of this prodrug starts with hydrolysis of the carboxylic ester group by an esterase

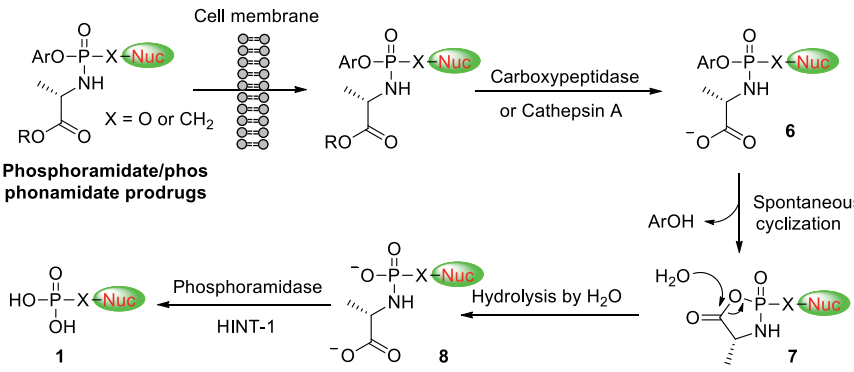
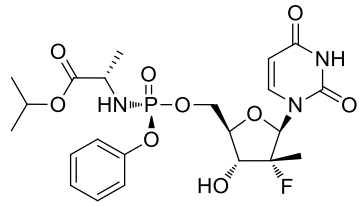


Figure 1.19 Activation of phosphoramidate/phosphonamidate prodrugs.

or cathepsin A to generate a carboxylate intermediate (**6**). Intramolecular cyclization of carboxylate **6** leads to a five-membered cyclic intermediate (**7**) by releasing a phenol molecule. Then, water-mediated ring-opening of compound **7** forms a diacid intermediate (**8**) and finally, cleavage of the amino acid moiety from compound **8** by intracellular phosphoramidase or the histidine triad nucleotide-binding protein 1 (HINT-1) produces the nucleoside monophosphate (**1**).

1.2.4.1. Sofosbuvir Sofosbuvir (**PSI-7977**, Sovaldi, Figure 1.20) is the first marketed prodrug developed *via* the ProTide approach and is used to treat patients infected with hepatitis C virus (HCV). Sofosbuvir is on the World Health Organization (WHO) model list of essential medicines [44]. After cellular uptake into hepatocytes, the prodrug undergoes the metabolism process illustrated in Figure 1.19 to form the pharmacologically active nucleoside triphosphate (NTP) [45]. The bioactive NTP will exert its action on HCV NS5B polymerase, which is responsible for the viral genome replication. By inhibiting HCV NS5B polymerase, sofosbuvir causes RNA chain termination and eventually stops the HCV replication [46].

The design rationale of the prodrug **PSI-7977** [46] is illustrated in Figure 1.21. Previous studies indicated that although its triphosphate (**9-TP**) is a potent HCV NS5B inhibitor [47–49], the parent nucleoside, 2′-fluoro-2′-*C*-methyluridine (**9**), is inactive in HCV replicon assays. Metabolism studies demonstrated that 2′-fluoro-2′-*C*-methylcytidine (**10**) can be metabolized to both the active triphosphate (**10-TP**) and the inactive 2′-fluoro-2′-*C*-methyluridine (**9**). Metabolism of the cytidine monophosphate (**10-MP**) leads to the monophosphate of uridine (**9-MP**), which could be further phosphorylated to the active uridine triphosphate (**9-TP**). However, 2′-fluoro-2′-*C*-methyluridine (**9**) cannot be directly metabolized into its monophosphate (**9-MP**) and this could be the reason why the uridine (**9**) itself is inactive. With a design based on these results, the nucleoside monophosphate (**9-MP**) prodrug may overcome the inefficient monophosphorylation step of the parent compound (**9**) to achieve high levels of the desired triphosphate (**9-TP**) in the liver.



Sofosbuvir
(PSI-7977)

Figure 1.20 Structure of sofosbuvir (**PSI-7977**).

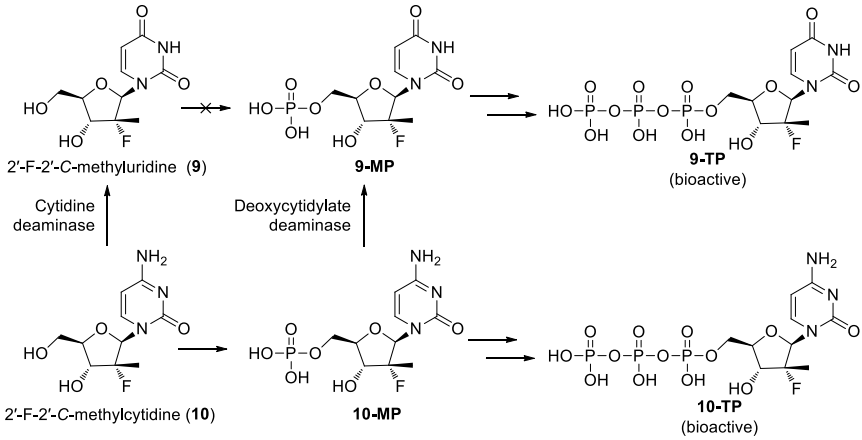
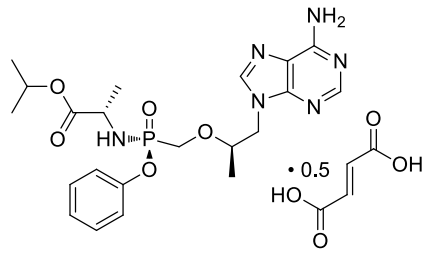


Figure 1.21 The design rationale of sofosbuvir (**PSI-7977**).

1.2.4.2. Tenofovir Alafenamide Fumarate Tenofovir alafenamide fumarate (TAF, **GS-7340**, Figure 1.22) is a new, orally available prodrug of the acyclic nucleotide analog tenofovir developed *via* the ProTide strategy. This prodrug has been approved by FDA [50] as a nucleotide reverse transcriptase inhibitor (Vemlidy) for the treatment of HIV-1 infection and chronic hepatitis B. Compared to the previously approved prodrug TDF, TAF exhibits significantly increased antiviral activity with higher stability *in vivo* and better lymphoid-tissue distribution [51]. The higher intracellular levels but lower plasma levels of TAF could be the reason for the fewer TAF-associated side effects compared to TDF [52]. After the cellular uptake, this ProTide is metabolized into the pharmacologically active tenofovir diphosphate (DP), which then results in the DNA chain termination, thus inhibiting the viral replication [53].



Tenofovir alafenamide fumarate (TAF)

Figure 1.22 Structure of tenofovir alafenamide fumarate (TAF).

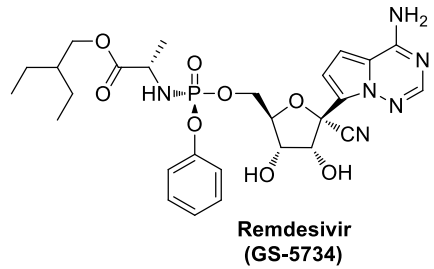


Figure 1.23 Structure of remdesivir (**GS-5734**).

1.2.4.3. Remdesivir Remdesivir (**GS-5734**, Figure 1.23) is a *C*-nucleoside, 1′-cyano-4-aza-7,9-dideaza adenosine prodrug bearing a phenyl 2-ethylbutyl L-alanine ester phosphoramidate moiety. This ProTide prodrug (Veklury) was first developed as a potential antiviral agent for the treatment of Ebola *via* intravenous administration [54, 55]. **GS-5734** also displayed a broad spectrum antiviral activity against RNA viruses including arenaviruses, coronaviruses, and filoviruses. During the COVID-19 pandemic, it was originally granted an FDA Emergency Use Authorization (EUA) for use in patients with suspected or confirmed COVID-19 in May 2020 and received full approval in October 2020 to treat COVID-19. In addition, remdesivir has been approved or authorized in some 50 countries as an emergency treatment of COVID-19.

1.2.4.4. AT-527 **AT-527** (Figure 1.24) is a hemisulfate salt of a 2′-fluoro-2′-*C*-methylguanosine phosphoramidate prodrug. As a novel HCV NS5B polymerase inhibitor, **AT-527** possesses potent *in vitro* anti-HCV activity with favorable preclinical profiles [56, 57]. This ProTide prodrug displayed about 10-fold higher antiviral activity than sofosbuvir against laboratory strains as well as clinical isolates of HCV genotypes 1–5. It is fully active against S282T-resistant variants with up to 58-fold higher potency than sofosbuvir. No *in vitro* toxicity was observed at concentrations up to 100 μM. **AT-527** was predominantly metabolized into the active nucleoside triphosphate (NTP) in

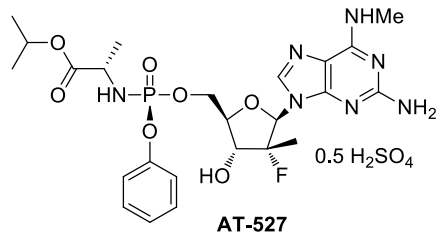


Figure 1.24 Structure of **AT-527**.

hepatocytes from multiple species. Consistently, it led to high levels of NTP in rats and monkeys after oral administration. This prodrug has exhibited clinical tolerability and potent antiviral activity in HCV-infected patients with a favorable human PK profile. **AT-527** interferes with viral RNA polymerase, a key enzyme in the replication of enveloped, positive single-stranded RNA viruses, including flaviviruses and coronaviruses. It has also demonstrated antiviral activity against SARS-CoV-2 infection and is currently under evaluation in clinical development for the treatment of COVID-19.

1.2.4.5. GS-6620 **GS-6620** (Figure 1.25) was the first *C*-nucleoside derivative reported as an HCV polymerase inhibitor with antiviral response in hepatitis C patients [58, 59]. It is a ProTide prodrug of 1′-cyano-2′-*C*-methyl 4-aza-7,9-dideaza adenosine with the 3′-OH masked by the isobutyryl group. **GS-6620** has also been developed as a potential treatment for other infectious diseases caused by viruses such as Ebola [60, 61].

1.2.4.6. NUC-1031 **NUC-1031** (Figure 1.26) is a ProTide prodrug of gemcitabine and a new promising anticancer reagent [62]. The activation of this prodrug is less dependent on deoxycytidine kinase and nucleoside transporters than the nucleoside gemcitabine. **NUC-1031** is resistant to cytidine

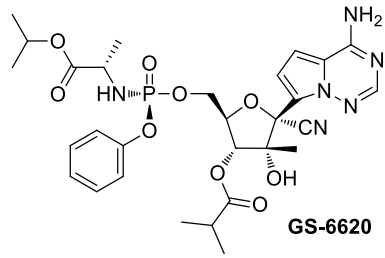


Figure 1.25 Structure of **GS-6620**.

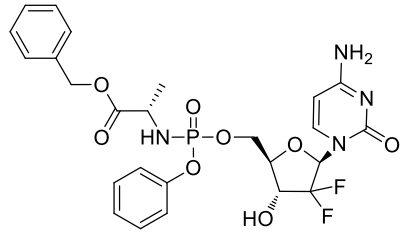


Figure 1.26 Structure of **NUC-1031**.

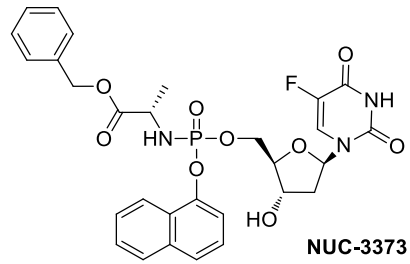


Figure 1.27 Structure of **NUC-3373**.

deaminase-mediated degradation and displays a significant inhibitory effect on tumor growth in pancreatic cancer xenograft models.

1.2.4.7. NUC-3373 **NUC-3373** (Figure 1.27) is another potential anticancer agent developed *via* the ProTide technology. It is a phosphoramidate prodrug of 5-fluoro-2′-deoxyuridine (FDUR) and was discovered by McGuigan *et al*. [63] In an extensive structure–activity relationship (SAR) investigation of 39 ProTide analogs, the amino acid ester region exhibited little contribution to the activity of this type prodrug. Replacement of the phenol moiety in the ProTide with 1-naphthalenol significantly improved the potency of the compound. Eventually, the *O*-naphthyl L-alanine benzyl ester ProTide (**NUC-3373**) was selected for further development.

NUC-3373 is the first drug candidate reported to overcome the key drug resistance associated with 5-fluorouracil (5-FU) and FDUR [64]. Thymidine kinase inhibition resulted in the reductions in the cytotoxic activity of the ProTide prodrug by a factor of only 4 and of the parent nucleoside FDUR by a factor of 136. Nucleoside transport inhibition mildly decreased cytotoxicity of **NUC-3373** (*vs* a 63-fold reduction in the cytotoxicity of FUDR). In an assessment of sensitivity to dihydropyrimidine dehydrogenase (DPD) degradation, **NUC-3373** concentration was not affected while the 5-FU concentration was significantly increased in an experiment with 5-FU. These results indicate that the prodrug is more independent of thymidine kinase and nucleoside transporters and also is not a substrate for DPD metabolism. **NUC-3373** displayed greater inhibitory effect on tumor growth (47%) than 5-FU (25%) in a colorectal cancer xenograft model.

1.2.4.8. Stampidine Stavudine is an FDA-approved anti-HIV/AIDS drug. The first phosphorylation of this nucleoside analog, however, is rate-limiting during the formation of the bioactive triphosphate metabolite [65]. Application of the ProTide approach to stavudine led to the discovery of stampidine (Figure 1.28), a ProTide prodrug bearing a *para*-bromophenyl L-alanine methyl ester moiety [66]. Stampidine displayed potent antiviral activity against both the wild-type HIV-1 strains and drug-resistant viral strains [66, 67]. Incorporation

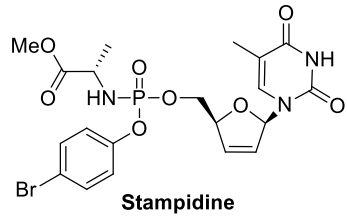


Figure 1.28 Structure of stampidine.

of an electron-withdrawing atom, bromine into the *O*-phenyl group can accelerate the metabolism of the ProTide to form the active triphosphate species. Stampidine is well tolerated in mice and rats at a dose of up to 500 mg/kg *via* intraperitoneal or oral administration [68]. This prodrug has been advanced into clinical development stages and is also considered to be a promising agent for pre-exposure prophylaxis against HIV infection [69].

1.2.4.9. Thymectacin Thymectacin, also known as **NB-1011** [70], (Figure 1.29) is a ProTide prodrug of brivudine (BVdU), an antiviral drug used to treat herpes zoster (shingles). Interestingly, this prodrug possesses potent anticancer activity and targets tumor cells with high levels of thymidylate synthase (TS) [71]. **NB-1011** displayed 10-fold higher cytotoxicity to 5-FU resistant and TS overexpressing colorectal tumor cells than to healthy cells. It is metabolized by TS into the nucleoside monophosphate (BVdU-MP), and BVdU-MP can compete with deoxyuridine monophosphate, the natural substrate, to bind to TS. The binding between BVdU-MP and TS is reversible, and thus TS can further convert BVdU-MP into cytotoxic metabolites. This prodrug has progressed into clinical trials for the treatment of colon cancer [72].

1.2.4.10. BMS-986094 **BMS-986094** (also known as **INX-08189**, Figure 1.30) is a double prodrug of anti-HCV 2′-β-*C*-methyl guanosine analog with a phosphoramidate group and a 6-*O*-methoxy purine base [73–75]. Both

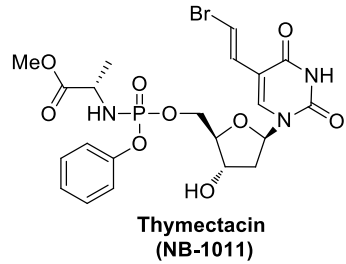


Figure 1.29 Structure of thymectacin.

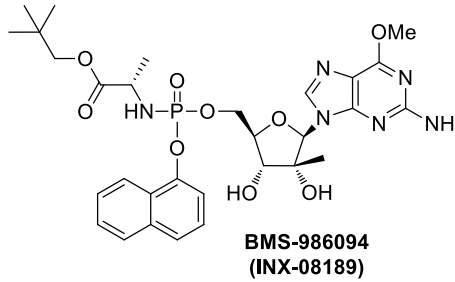


Figure 1.30 Structure of **BMS-986094** (**INX-08189**).

the prodrug moieties can increase the lipophilicity of the parent guanosine monophosphate. **INX-08189** displays a 500-fold better antiviral activity (EC_{50} = 10 nM) against the HCV genotype 1b subgenomic replicon than the parent compound, which is consistent with the intracellular levels of the active 2′-*C*-methyl guanosine triphosphate in primary human hepatocytes. Separated single isomers of the prodrug exhibited similar anti-HCV activity and both isomers were degraded by enzymes, showing similar half-lives. Therefore, the prodrug **INX-08189** was selected for clinical development to treat chronic HCV infection.

1.2.4.11. PSI-353661 **PSI-353661** (Figure 1.31) is also a double prodrug containing ProTide and 6-*O*-methoxy moieties for the treatment of HCV infection [76]. The parent nucleoside 2′-deoxy-2′-fluoro-2′-*C*-methylguanosine displayed only weak anti-HCV active (EC_{90} = 69.2 μM) in a replicon assay although its triphosphate is a potent HCV NS5B polymerase inhibitor (IC_{50} = 5.94 μM). Most of these prodrugs exhibited significantly improved antiviral activity (> 1000-fold) compared to the parent nucleoside. Among them, **PSI-353661** demonstrated high potency against both the wild type and the S282T-resistant replicon (EC_{90} = 0.008~0.011 μM) with no cytotoxicity at 100 μM concentration.

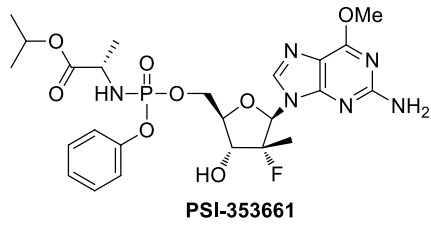
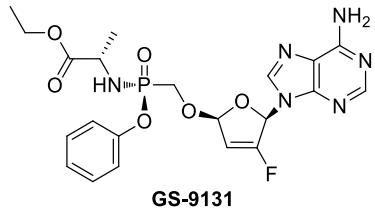


Figure 1.31 Structure of **PSI-353661**.

1.2.4.12. GS-9131 The parent compound of **GS-9131**, (5-(6-amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl)phosphonic acid (2′-Fd4AP, **GS-9148**) is a novel and potent nucleotide HIV-1 reverse transcriptase (RT) inhibitor against multiple NRTI-resistant HIV-1 variants [77, 78]. **GS-9131** (Figure 1.32) is an orally bioavailable ProTide prodrug of 2′-Fd4AP bearing an ethylalaninyl phosphoramidate moiety [78–80]. This phosphoramidate prodrug was designed as a substrate for the lysosomal carboxypeptidase (cathepsin A), which is highly expressed in peripheral blood mononuclear cells (PBMC). It also displayed favorable intestinal and hepatic stabilities, and thus can reach lymphoid cells efficiently after oral administration, achieving high levels of the bioactive diphosphate of the parent nucleotide.

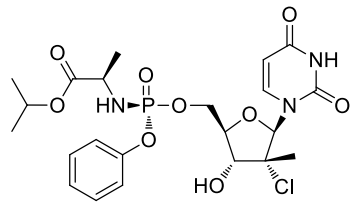
1.2.4.13. Uprifosbuvir Uprifosbuvir (**MK-3682/IDX21437**, Figure 1.33) is a potent antiviral ProTide prodrug candidate of 2′-chloro-2′-methyluridine [81, 82]. Unlike most S_P-configuration- and L-amino acid-based ProTide prodrugs, **MK-3682** is a R_P-isomer prodrug bearing a D-alanine isopropyl ester moiety [81] and has entered phase III clinical trials as a pan-genotypic inhibitor of HCV NS5B for the treatment of patients with HCV [83–85].

1.2.4.14. CL-096 Our laboratory reported a phosphoramidate prodrug of β-D-2′-deoxy-2′-α-fluoro-2′-β-*C*-(fluoromethyl)uridine [86–88] (**CL-096,** Figure 1.34). This novel difluoro uridine phosphoramidate prodrug possesses potent antiviral activity against HCV genotypes 1b, 1a, 2a, and S282T



GS-9131

Figure 1.32 Structure of **GS-9131**.



Uprifosbuvir (MK-3682)

Figure 1.33 Structure of uprifosbuvir (**MK-3682**).

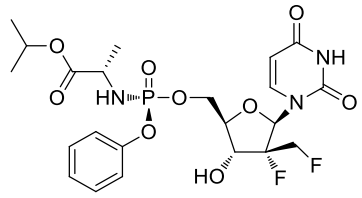
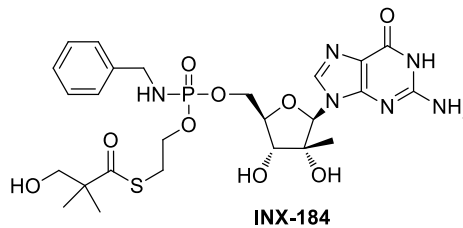


Figure 1.34 Structure of **CL-096**.

(EC_{50} = 0.18–1.13 μM) with no cytotoxicity observed at the highest tested concentration (1000 μM). **CL-096** is orally available, is well tolerated at a dose of 4 g/kg in mice, and produces a high level of the corresponding nucleoside triphosphate (NTP) in rat livers.

1.2.4.15. IDX-184 **IDX-184** (Figure 1.35) is a guanosine analog prodrug developed as a potent NS5B RNA polymerase inhibitor [89, 90] for the treatment of HCV infection. Unlike most ProTides, this phosphoramidate prodrug contains an *N*-benzyl group instead of the amino acid ester moiety and a masked 2-mercaptoethyl group instead of the *O*-aryl moiety. This drug candidate has progressed into clinical development and has displayed reasonable effectiveness in early clinical trials. In addition, **IDX-184** also showed antiviral activity against emerging viruses such as the Zika virus [91, 92], MERS-CoV [93], and SARS-CoV-2 [94, 95].

1.2.4.16. CL-206 Our group also developed a series of new phosphoramidate prodrugs by replacing the aryl group in ProTide with a substituted benzyl moiety. These novel phosphoramidates have a liver-targeting feature similar to that of HepDirect prodrugs, as the cleavage of the benzyl group requires cytochrome P450 (CYP) enzymes in the liver. Application of this prodrug approach to 5-fluoro-2′-deoxyuridine (FDUR) led to the discovery of **CL-206** (Figure 1.36) as a candidate drug for the treatment of hepatocellular carcinoma (HCC) [87, 96]. Biological evaluation including stability, activation rate, and cytostatic activity on a cancer cell line (SMMC7721) demonstrated that the optimal *O*-benzyl moiety is an *ortho*-methylbenzyl group. Compared to the racemic prodrug (**13**),



INX-184

Figure 1.35 Structure of **IDX-184**.

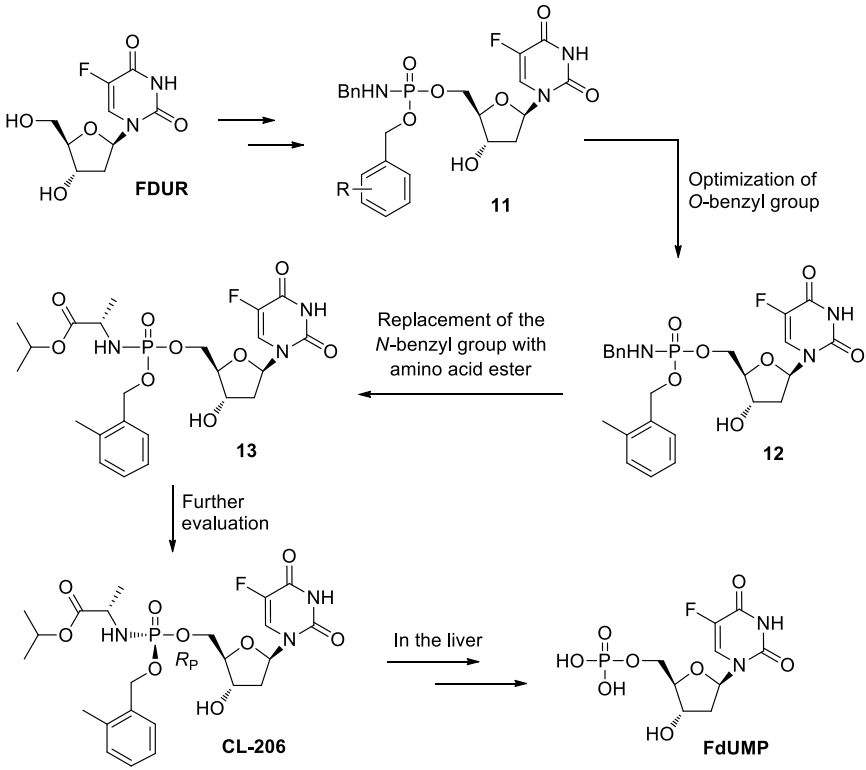


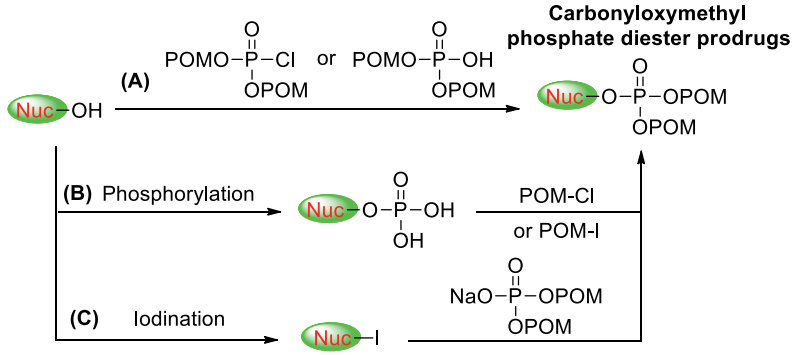
Figure 1.36 Discovery of **CL-206**, a new phosphoramidate prodrug with an *O*-benzyl group.

the R_p-enantiomer (**CL-206**) displayed equally good anticancer activity with slightly lower toxicity on a normal liver cell line (LO2). As expected, metabolism studies in rats revealed that **CL-206** can deliver the desired nucleoside monophosphate (FdUMP) to the liver with a high liver-targeting index. This prodrug is well-tolerated at an oral dose up to 3 g/kg in mice and exhibits a good inhibitory effect on tumor growth in a mouse xenograft model. These results suggest that **CL-206** is a potential new agent for anti-HCC therapy *via* oral administration.

1.3. SYNTHESIS OF NUCLEOSIDE PHOSPH(ON)ATE PRODRUGS

1.3.1. Synthesis of Carbonyloxymethyl Diester Prodrugs

Generally, there are three approaches to the development of nucleoside phosphate diester prodrugs bearing carbonyloxymethyl groups. Taking the preparation of bis(POM) nucleoside phosphates as an example, the synthetic methods include [3] (A) direct reactions of a nucleoside analog with the bis(POM)-phosphorochloridate in the presence of base, or with bis(POM)-phosphate *via*



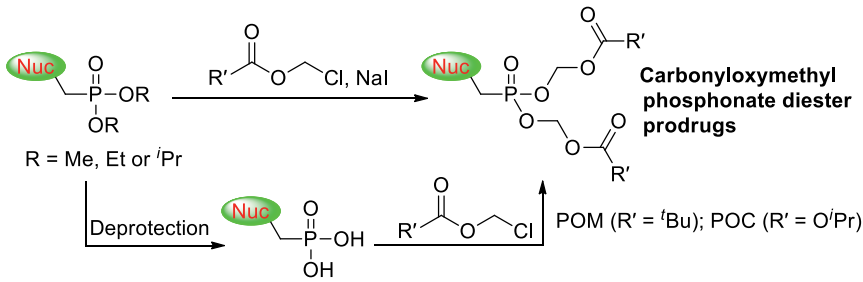
Scheme 1.1 Synthesis of carbonyloxymethyl phosphate diester prodrugs.

Mitsunobu coupling; (B) phosphorylation of a nucleoside followed by the reactions with a carbonyloxymethyl chloride or iodide; (C) iodination of the hydroxyl group in a nucleoside followed by replacement of the iodo group with a bis(POM)-phosphate salt (Scheme 1.1).

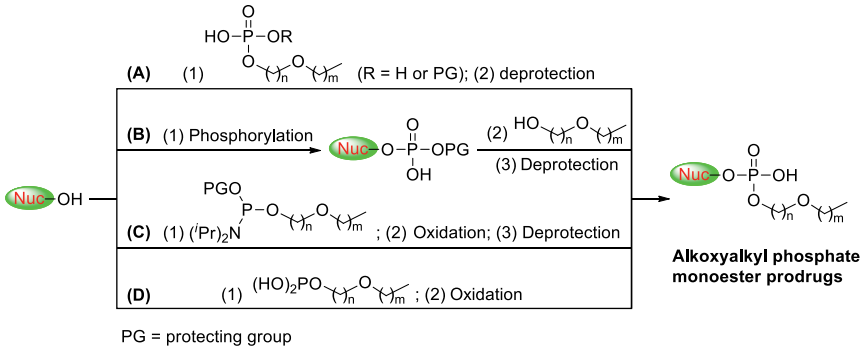
The preparation of nucleotide carbonyloxymethyl diester prodrugs is quite straightforward. Starting with nucleotide analogs protected with simple dialkyl groups [3], the expected prodrugs can be synthesized *via* a direct reaction with carbonyloxymethyl chloride reagents in the presence of sodium iodide or through phosphonate deprotection followed by the reaction with carbonyloxymethyl chlorides (Scheme 1.2).

1.3.2. Synthesis of Alkoxyalkyl Monoester Prodrugs

The synthesis of nucleoside alkoxyalkyl phosphate monoester prodrugs can be achieved from the corresponding parent compound *via* the following pathways [3]: (A) Mitsunobu coupling with an alkoxyalkyl phosphate reagent followed by deprotection, if necessary; (B) sequential phosphorylation, coupling with an alkoxyalkyl phosphate and deprotection; (C) coupling with an



Scheme 1.2 Synthesis of carbonyloxymethyl phosphonate diester prodrugs.

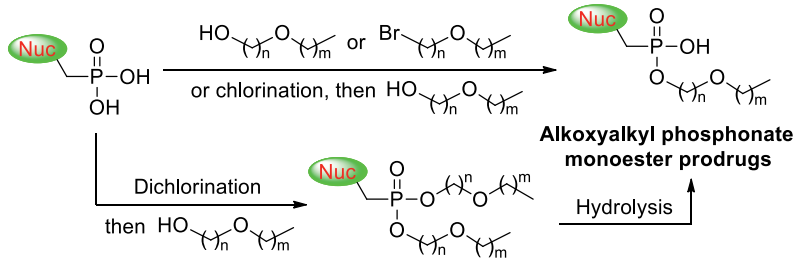


Scheme 1.3 Synthesis of alkoxyalkyl phosphate monoester prodrugs.

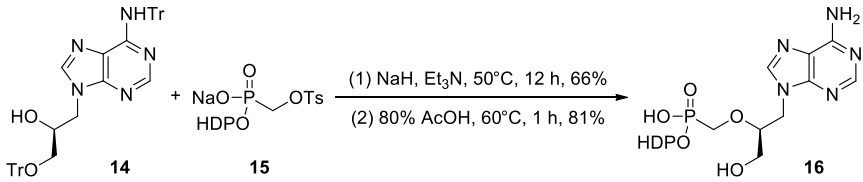
alkoxyalkyl phosphoramidite reagent followed by oxidation and deprotection; (D) condensation with a phosphite and then oxidation (Scheme 1.3).

Nucleotide alkoxyalkyl monoester prodrugs can be prepared [3] from the reaction of the nucleoside phosphonic acid with an alkoxyalkyl alcohol in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) or *via* Mitsunobu coupling. The parent nucleotide can also be directly alkylated by an alkoxyalkyl bromide or first activated through monochlorination followed by substitution with an alkoxyalkyl alcohol to access the prodrug. This kind of prodrug can also be synthesized by selective hydrolysis of the corresponding nucleotide alkoxyalkyl diester intermediate (Scheme 1.4).

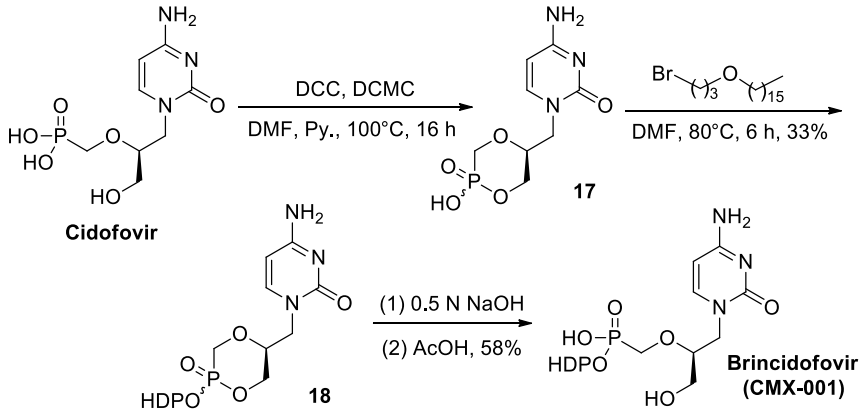
Hostetler *et al*. reported a concise synthesis of the (*S*)-3-hydroxy-2-(phosphono-methoxy)propyl (HPMP) adenine prodrug (**16**) by direct introduction of the monoalkoxyalkyl phosphonate monoester moiety with a tosylate intermediate already containing the HDP chain (**15**) [97] (Scheme 1.5). Brincidofovir (**CMX-001**), a prodrug of cidofovir, can be prepared *via* sequential intramolecular cyclization (**17**) of the parent nucleotide, alkylation with alkoxyalkyl bromide producing **18**, and finally hydrolytic ring-opening [98] (Scheme 1.6).



Scheme 1.4 Synthesis of alkoxyalkyl phosphonate monoester prodrugs.



Scheme 1.5 Synthesis of HPMP-adenine prodrug (**16**).



Scheme 1.6 Synthesis of prodrug brincidofovir (**CMX-001**).

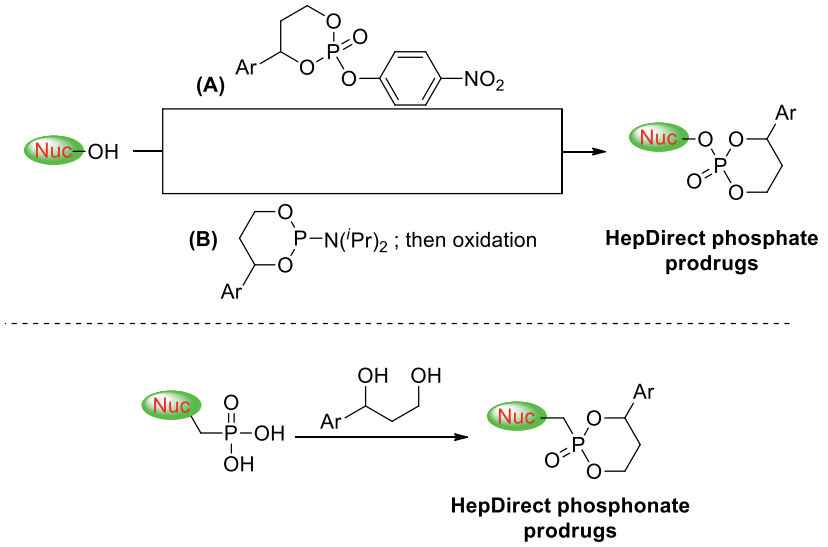
1.3.3. Synthesis of HepDirect Prodrugs

HepDirect phosphate prodrugs [8] can conveniently be synthesized by coupling the parent nucleoside with pre-prepared *p*-nitrophenyl phosphate intermediates or P(III) phosphoramidite intermediates followed by oxidation. Preparation of HepDirect phosphonate prodrugs [38] can be achieved through direct coupling with 1-arylpropane-1,3-diol (Scheme 1.7).

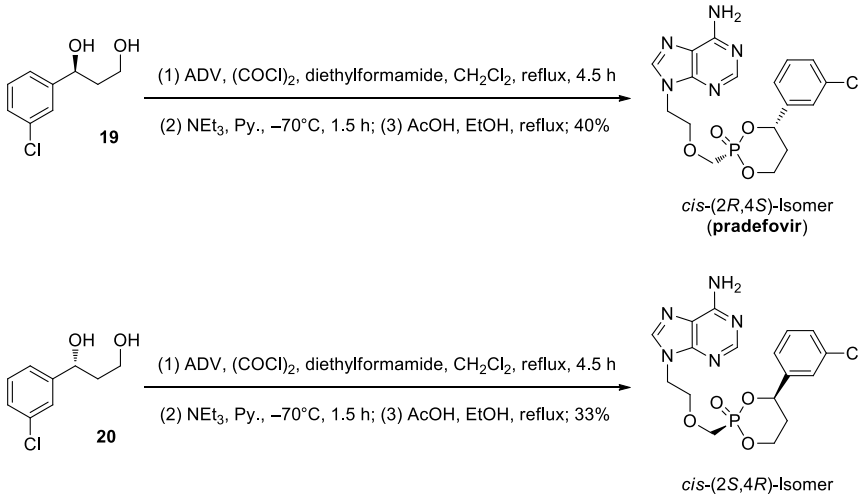
Because of there are two chiral centers in the HepDirect prodrugs moiety, there could be four diastereoisomers. Using enantiomerically pure propane-1,3-diols as starting materials will lead to the formation of only two of the diastereoisomers, either the *cis*- or the *trans*-isomers. Under optimized reaction conditions, pradefovir and the other *cis*-isomer can even be synthesized enantioselectively from the corresponding diols **19** and **20**, respectively [38] (Scheme 1.8).

1.3.4. Synthesis of Phosphoramidate and Phosphonamidate Prodrugs

Generally, the phosphoramidate prodrugs can be accessed by any of three pathways [3]: (A) pre-formation of a phosphoramidate intermediate already containing an aryl, amino acid ester and a leaving group, followed by substitution of

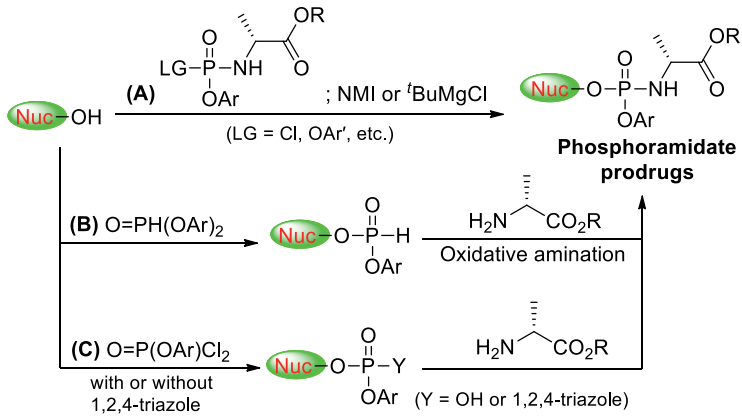


Scheme 1.7 Synthesis of HepDirect phosph(on)ate prodrugs.

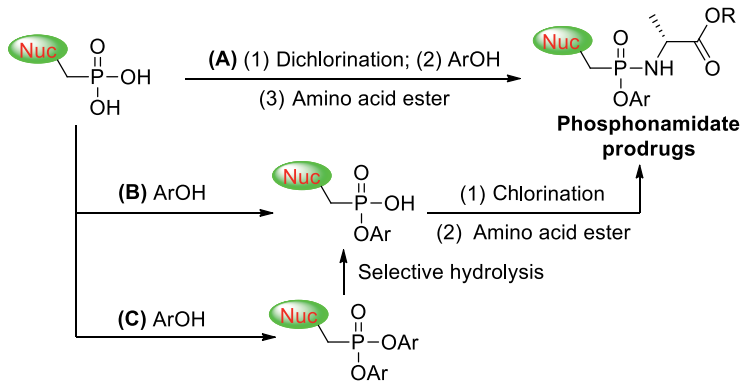


Scheme 1.8 Enantioselective synthesis of pradefovir and the other *cis*-isomer.

the parent nucleoside in the presence of *N*-methylimidazole (NMI) or *tert*-butylmagnesium chloride (^{t}BuMgCl); (B) sequential substitution of a diarylphosphite [O=PH(OAr)$_2$] by the nucleoside and oxidative amination with an amino acid ester; (C) coupling of the nucleoside with an aryl phosphorodichloridate, followed by reaction with an amino acid ester (Scheme 1.9).



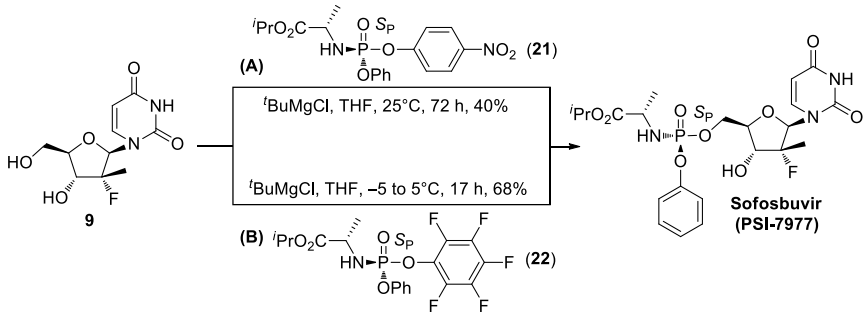
Scheme 1.9 Synthesis of phosphoramidate prodrugs.



Scheme 1.10 Synthesis of phosphonamidate prodrugs.

The preparation of phosphonamidate prodrugs can be achieved [3] by (A) dichlorination of the parent nucleotide and subsequent substitution with first a phenol and then an amino acid ester; or (B and C) chlorination of a pre-formed nucleotide monoaryl phosphonate followed by coupling with an amino acid ester (Scheme 1.10).

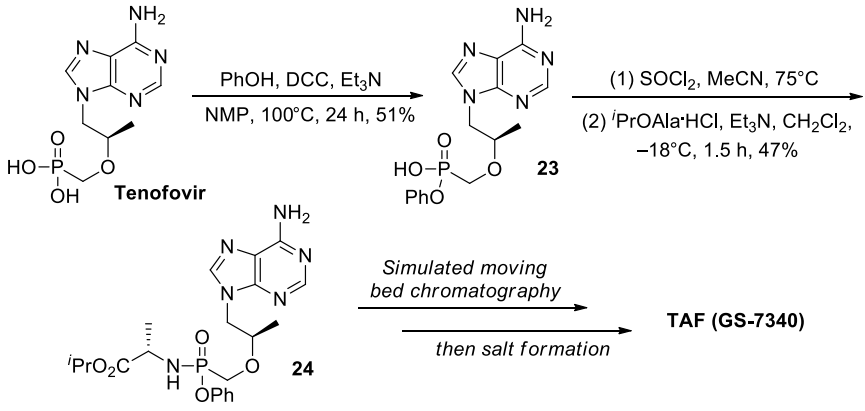
In most cases, the S_P and R_P isomers of phosphoramidate and phosphonamidate prodrugs display different biological profiles, and this promotes the development of diastereoselective approaches to access these types of prodrugs. One of the most frequently used methods is use of pre-formed chiral aryloxy phosphoramidate intermediates (see Method A in Scheme 1.9). For example, Ross *et al*. [99] reported the synthesis of sofosbuvir (**PSI-7977**) using chiral phosphoramidate reagents with substituted phenols as leaving groups (Scheme 1.11). Such phosphoramidate reagents can be readily prepared from commercially



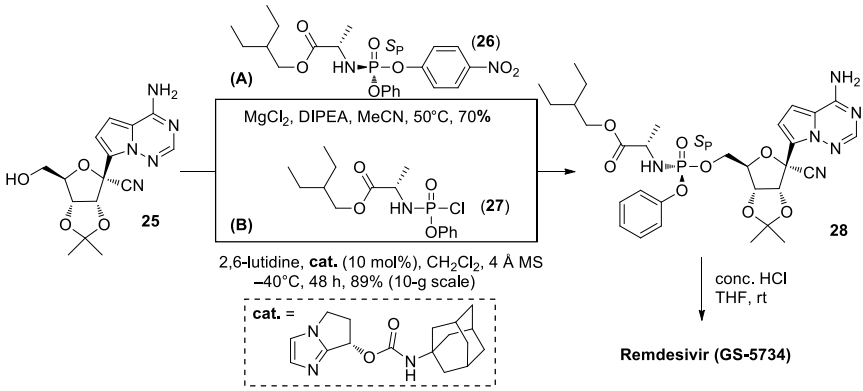
Scheme 1.11 Asymmetric synthesis of sofosbuvir (**PSI-7977**).

available substituted aryl dichlorophosphates by sequential reactions with phenol and then with an amino acid ester. The reaction of 2′-fluoro-2′-methyluridine with the S_p-isomer of *p*-nitrophenyl intermediate **21** in the presence of ^{t}BuMgCl followed by two recrystallizations from CH_2Cl_2 produced **PSI-7977** in 40% yield (Scheme 1.11A). Replacement of the *p*-nitrophenyl group in the phosphoramidate reagent with a pentafluorophenyl group (**22**) resulted in the formation of the desired prodrug in a better yield after two recrystallizations (68%, Scheme 1.11B).

As the parent nucleotide tenofovir already possesses a phosphonic acid group, the chiral ProTide prodrug TAF (**GS-7340**) cannot be prepared using the above strategy. Chapman *et al*. developed a practical method for the kilogram-scale preparation of the free base of this prodrug [100]. Starting with the parent nucleoside tenofovir, a DCC-mediated coupling reaction with phenol in *N*-methyl pyrrolidone (NMP) selectively masked the first hydroxyl group (**23**). The remaining hydroxyl group is chlorinated with sulfurous dichloride ($SOCl_2$) and then further substituted by the amino acid isopropyl ester moiety. Finally, the diastereomerically pure prodrug (**24**) was isolated by simulated moving bed chromatography (Scheme 1.12).



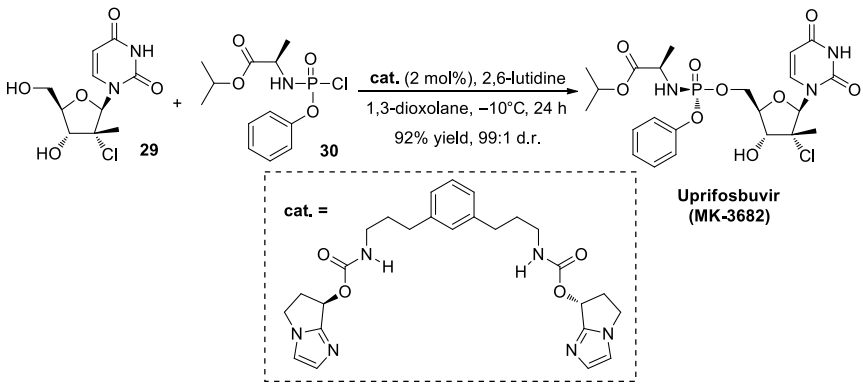
Scheme 1.12 Preparation of tenofovir alafenamide fumarate (**GS-7340**).



Scheme 1.13 Asymmetric synthesis of remdesivir (**GS-5734**).

Remdesivir (**GS-5734**) was previously synthesized [101] employing the single S_P-isomer of a *p*-nitrophenoxy phosphoramidate precursor (**26**) with the protected parent adenine *C*-nucleoside (**25**) in $MgCl_2$ and *N*,*N*-diisopropylethylamine (DIPEA) followed by deprotection (Scheme 1.13A). Recenty, Wang *et al*. [102] reported a catalytic asymmetric synthesis of this anti-COVID-19 drug in 96% yield from the P-racemic phosphoryl chloride (**27**) and the protected parent nucleoside (**25**) with excellent stereoselectivity (22 : 1 S_P:R_P). This dynamic kinetic asymmetric transformation (DyKAT) was achieved by utilization of a chiral imidazole catalyst. This catalyst contains a bicyclic imidazole skeleton and an adamantinyl-substituted carbamate group, which are both required for the DyKAT (Scheme 1.13B).

DiRocco *et al*. [82] reported a multifunctional catalyst for stereoselective synthesis of phosphoramidate prodrugs such as uprifosbuvir (**MK-3682**) from the P-racemic phosphoryl chloride (**30**). The rational catalyst design from mechanistic studies and computational modeling led to a stereoselective synthesis up to 99 : 1 (Scheme 1.14).



Scheme 1.14 Catalytic asymmetric synthesis of uprifosbuvir (**MK-3682**).

1.4. CONCLUSION

Prodrug strategies for phosphates or phosphonates have significantly facilitated the drug discovery and development of nucleotide and nucleoside analogs. They can increase the potency of a parent compound and even reveal the activity of an inactive nucleos(t)ide analog. The phosph(on)ate prodrugs usually display better bioavailability than the associated parent molecule mainly because they have improved lipophilicity. Some nucleos(t)ides, traditionally administered only by injection, may be developed into orally available drugs through these prodrug approaches. However, there are still some challenges, such as efficient methods for enantioselective phosph(on)ate prodrug synthesis and tissue-targeted drug delivery. Given the advantages and achievements of phosph(on)ate prodrugs, this synthetic branch may develop potential drug candidates against emerging virus infections for which no treatment is currently available, and it will further explore the applications of the phosph(on)ate prodrug strategies in non-nucleoside drug discovery.

ABBREVIATIONS

3TC	lamivudine
5-FU	5-fluorouracil
ADP	adefovir dipivoxil
ADV	adefovir
AIDS	acquired immune deficiency syndrome
AZT	zidovudine
BVdU	brivudine
CC_{50}	50% cytotoxic concentration
CDV	cidofovir
CFR	cholesterol-fed rat
COVID-19	corona virus disease 2019
CYP	cytochrome P450
DCC	*N,N′*-dicyclohexylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DNA	deoxyribonucleic acid
DP	diphosphate
DPD	dihydropyrimidine dehydrogenase
DyKAT	dynamic kinetic asymmetric transformation
EC_{50}	50% effective concentration
EC_{90}	90% effective concentration
EUA	Emergency Use Authorization
FDA	the US Food and Drug Administration
FdUMP	5-fluoro-2′-deoxyuridine monophosphate
FDUR	5-fluoro-2′-deoxyuridine

FNC	azvudine
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDP	hexadecyloxypropyl
HINT-1	histidine triad nucleotide-binding protein 1
HIV	human immunodeficiency virus
HPMP	(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl
HSV	herpes simplex virus
IC_{50}	50% inhibitory concentration
LPC	lysophosphatidylcholine
MERS-CoV	Middle East respiratory syndrome coronavirus
MP	monophosphate
NMI	*N*-methylimidazole
NMP	*N*-methyl pyrrolidone
nRTI	nucleotide analog reverse transcriptase inhibitor
NS5B	non-structural protein 5B
NTP	nucleoside triphosphate
ODE	octadecyloxyethyl
PA	phosphonic acid
PBMC	peripheral blood mononuclear cell
PG	protecting group
PK	pharmacokinetics
PMEA	9-(2-phosphonomethoxyethyl)adenine
PMPA	(*R*)-9-(2-phosphonylmethoxypropyl)adenine
POC	isopropyloxymethyl
POM	pivaloyloxymethyl
RT	reverse transcriptase
SAR	structure–activity relationship
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
$t_{1/2}$	half-life
TAF	tenofovir alafenamide fumarate
TDF	tenofovir disoproxil fumarate
TP	triphosphate
TR	thyroid hormone receptor
TS	thymidylate synthase
WHO	World Health Organization

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