Novel Potential Anticancer Naphthyl Phosphoramidates of BVdU: Separation of Diastereoisomers and Assignment of the Absolute Configuration of the Phosphorus Center

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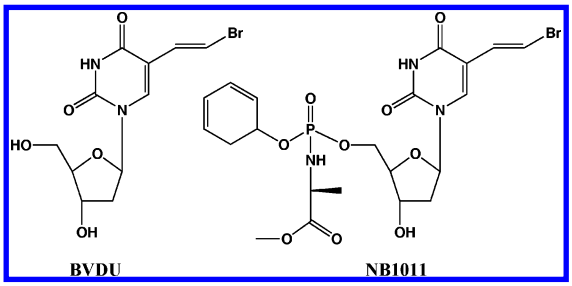
Abstract: We have previously reported our SAR optimization of the anticancer agent thymectacin. Tuning of the parent ProTide structure initially involved the amino acid and, subsequently, the aromatic masking group on the phosphate moiety. Herein, derivatives bearing the combined modifications are reported and biological evaluation is described. Moreover, separation of the diastereoisomeric final product mixture shows a different cytostatic activity for the two diastereoisomers. Through computational and NMR studies, the absolute stereochemistry of the phosphorus center of the two diastereoisomers has been suggested.

Nucleoside analogues represent an extremely effective tool for the treatment of cancer and viral infections. For these compounds, the action of kinases is required to convert the inactive nucleoside into biologically active nucleotide (mono-, di-, and triphosphate). Unfortunately, the dependence on kinases significantly limits the biological profile of nucleoside analogues because of their high specificity toward substrates and, no less important, a lower expression of these enzymes often leads to emergence of resistance to the nucleoside treatment. Due to the polarity of nucleotides themselves, circumventing kinase activation problems cannot be achieved with direct administration of the preformed free phosphate, as the resulting cell penetration would be too poor to show any significant therapeutic effect.

The phosphoramidate approach was introduced by McGuigan et al. in 1992[1] as a means to improve cellular penetration of nucleotides and to bypass the first step of kinase-mediated activation of nucleosides. Our method has been applied by both our lab and others to a wide variety of nucleosides and, nowadays, is recognized as one of the most successful approaches for the delivery of nucleoside monophosphates inside cells.[2]

Our technology has recently led NewBiotics to NB1011[3] (thymectacin), an aryloxy phosphoramidate derivative of BVdU (brivudin), which entered clinical evaluation against colon cancer.[4] On the basis of our experience of widespread phosphoramidate modifications, we first prepared a new series of phenyl phosphoramidates related to thymectacin, tuning the phenyl, ester, and amino acid regions, observing significant enhancements in activity versus three different tumor cell lines.[5] Because lipophilicity might play a crucial role for the delivery of ProTides inside cells,[6] new analogues have been designed focusing on the introduction of naphthyl as new aromatic masking group on the phosphate moiety, which has led to a further increase in cytostatic activity against a panel of cancer cell lines.[7a,b]



The present work describes the synthesis and biological evaluation of a new family of BVdU ProTides, combining the modifications on the ester and amino acid moieties with the use of the new aryl group on the phosphate, naphthyl. The main objectives of this study were (i) to enhance the cytostatic potency and (ii) to further investigate the structure−activity relationship within BVdU phosphoramidates.

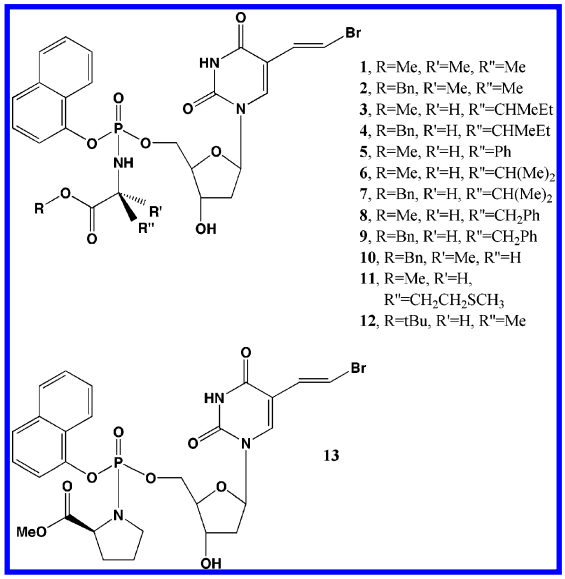
The compounds involved with our study are shown in Scheme 1. The target ProTides were all prepared in one step from BVdU using the phosphorochloridate chemistry we have extensively described.[8,9]

The synthesis involves phosphorylation of 1-naphthol with phosphorus oxychloride, followed by the coupling with different esterified amino acid salts to give naphthyloxy-phosphorochloridates, which were generally purified by flash chromatography and then coupled with BVdU in the presence of 1-methylimidazole (NMI). Yield of the final purified compounds resulted in a 6.5−49.7% range. The general synthetic route is reported in Scheme 2.

Due to the stereochemistry at the phosphorus center, the final compounds isolated from the coupling to BVdU are diastereoisomers. The confirmation of the presence of two diastereoisomers is shown by P-31 (two signals, 1:1 ratio), H-1, and C-13 NMR.

Compounds **1**−**13** were evaluated for their cytostatic activity against a panel of different tumor cell lines in vitro: MDA

Scheme 1. Structures of **1**−**13**



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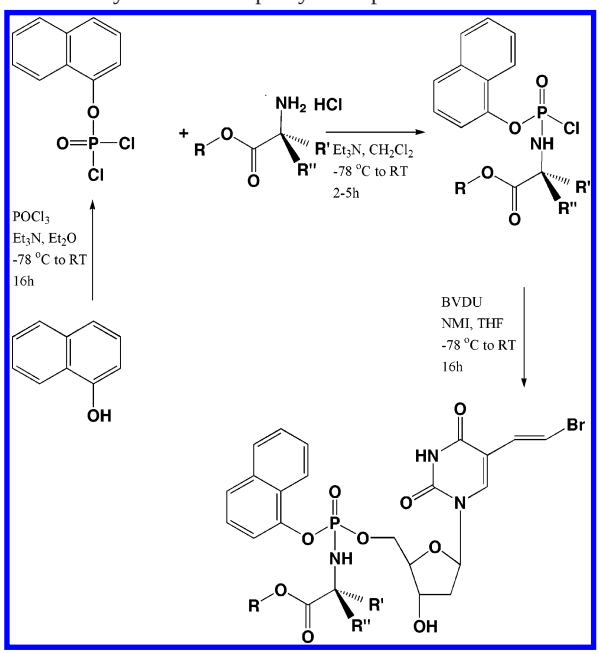
Scheme 2. Synthesis of Naphthyl Phosphoramidates



MB231 (breast cancer) and PC-3 (prostate cancer) for entries **1**−**5** and thymectacin; T24 (bladder cancer) was also considered for entries **6**−**13**. Data are reported in Table 1.

According to the data shown in Table 1, most of the naphthyl phosphoramidates synthesized appear to be significantly more active than thymectacin, which displays only a moderate activity in our in vitro evaluations. Moreover, the L-alaninyl benzyl ester naphthyloxy-phosphoramidate (CPF98) which has been previously reported by us has been added to Table 1.[7a−b]

For the prostate cancer cell line, lipophilicity seems to play a fundamental role for the biological activity of these ProTides: the activity of compounds bearing the same amino acid moiety (dimethylglycine: **1**, **2**; L-isoleucine **3**, **4**; L-valine: **6**, **7**) is boosted every time the ester group is changed from methyl to benzyl, leading to a 100-fold increase versus thymectacin (entries **2** and **4**). Not surprisingly, also the presence of a highly lipophilic amino acid as phenylglycine leads to a significant activity (compound **5**).

On the other hand, this trend is not applicable to the breast cancer cell line. Activation of phosphoramidates has been reported to be dependent on the action of esterases and phosphoramidase activities;[8−10] it is possible that a different enzymatic activity in the two cell lines is responsible for the differences observed in the biological data. Further assays are underway to probe this aspect. Nevertheless, naphthyl phosphoramidates did show noteworthy activities also versus the breast cancer cell line, in particular compounds **2**, **3**, and **9** have achieved between 30- and 50-fold increase in potency versus thymectacin. Moreover, compound **1**, showing a 250-fold boost versus this particular cancer cell line, becomes the most active BVdU-related phosphoramidate with cytostatic activity against a breast cancer cell line reported. Significantly, the corresponding 'phenyl' ProTide reported previously by us displays an EC_{50} of 41.1 μM in the same assay.[5]

Last, tuning the structure of the lead ProTide by introducing the naphthyl moiety and subsequently modifying the amino acid core has led to an enhancement of potency in all the three cell lines, such as compound **1** for the breast cancer, **4** and **5** for the prostate, and **13** for the bladder cancer cell line.

Table 1. Cytostatic Effect of Test Compounds (EC_{50}/μM)

compound	amino	ester	breast MDAMB231	prostate PC-3	bladder T24
NB1011	L-Ala	Me	79	155	−
CPF98	L-Ala	Bn	15.2	1.7	−
1	Me_2Gly	Me	0.32	65.9	−
2	Me_2Gly	Bn	2.7	1.5	−
3	L-Ile	Me	1.5	6.9	−
4	L-Ile	Bn	130	1.4	−
5	L-PhGly	Me	105	1.7	−
6	L-Val	Me	14.8	15.8	43.5
7	L-Val	Bn	5.9	8.3	12.7
8	L-Phe	Me	8.5	10.2	5.3
9	L-Phe	Bn	1.96	5.8	269
10	D-Ala	Bn	6.3	6.1	2.8
11	L-Met	Me	28.1	44.6	19.6
12	L-Ala	tBu	4.8	11	4
13	L-Pro	Me	6.5	10.5	0.4

isomers (1:1 ratio). Previous work carried out by Saboulard et al.[11] in 1999 indicates that carboxyl ester cleavage is a fundamental step for the activation of phosphoramidates. Enzymatic stability in the extracellular environment (i.e. plasma) and in different cellular preparations was found to be stereospecific with large and unpredictable differences in stereoselective metabolic rate noted by Siccardi et al.[12] Separation of phosphoramidate diastereoisomers by column chromatography has been shown to be problematic and even by using HPLC preparative methods, it remains a hard task to achieve.[13] Furthermore, when single diastereoisomers have been isolated, identification of the corresponding absolute stereochemistry has never been elucidated, leaving HPLC retention time and ^{31}P NMR chemical shift as the only parameters to discriminate between the two isomers.

In the case of compound **10**, the mixture has been reasonably separated on reverse phase and the two diastereoisomers were tested against the MDA MB231 cell line (breast cancer). The fast eluting diastereoisomer (**fe**) emerged as slightly less active than the mixture (compd **10**), with an EC_{50} of 7.4 μM. On the other hand, the slow eluting diastereoisomer (**se**) is about 10 times more active than the mixture, showing an EC_{50} of 0.5 μM. The higher activity could be due to a better diffusion through cell membranes,[14] the slow eluting diastereoisomer being the more lipophilic, or to a more efficient stereoselective metabolism of the **se** diastereoisomer. Biological evaluation against the other different cancer cell lines is in progress.

Given this striking result, our major interest has been to find out a method to attribute the corresponding absolute stereochemistry to each of the two diastereoisomers.

The slow eluting (**se**) diastereoisomer shows downfield shifts (between 0.1 and 0.2 ppm) on H NMR for the H-5b, H-6, and H-2′ protons compared to the fast eluting (**fe**) isomer. Owing to the presence of three aromatic systems (nucleoside analogue base, phenyl and naphthyl), the former protons' chemical shifts might be perturbed by an anisotropic effect. The methylene protons of the benzylic ester display a more striking difference: for the **fe** diastereoisomer, a double doublet results as the main feature of their signal (traces of the other isomer are present) while, for the **se** diastereoisomer, the two protons couple with each other and show an AB-system (Figure 1).

Conformational studies were performed using the Sybyl 7.0 software package,[15] which allowed the identification of a series of distinct low energy conformations. The lowest energy conformation found for each diastereoisomer is shown in Figure

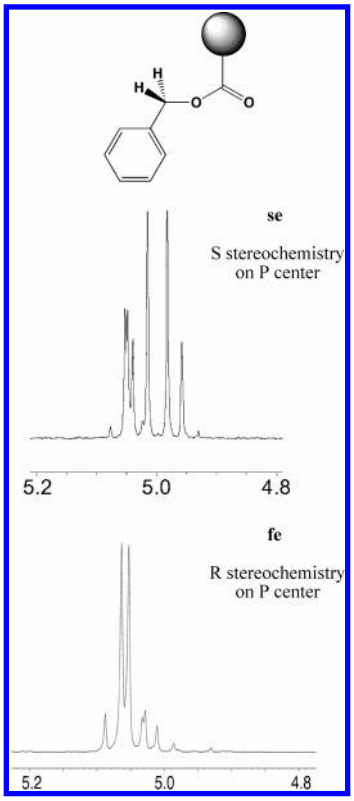


Figure 1. H NMR spectra of the methylene protons of the benzylic ester moiety of the isolated diastereoisomers of compound **10**.

For the *S* diastereoisomer (for clarity the two diastereoisomers are named after the absolute stereochemistry of the corresponding phosphorus center) the three aromatic moieties are stacked in pi-pi interactions where the naphthyl lays between the phenyl group and the nucleoside base. In this case, chemical shift changes are reasonable to appear for the protons closer to such an extended pi-electron cloud (e.g. H-5b, H-6 and H-2′). Furthermore, the apparent rigidity of this conformation, conferred by the described aromatic interactions, justifies the observed NMR pattern for the methylene hydrogens of the benzylic ester, which became nonequivalent.

The *R* conformation does not show any pi-pi interaction among the aromatic rings as in the case of the *S* diastereoisomer, and the greater flexibility around the methylene group of the ester moiety reduces the magnetic differences between the two diastereotopic protons. Therefore, by combining the NMR and the conformational data, we can propose the *S* phosphorus absolute configuration to the slow eluting (more lipophilic) diastereoisomer and, consequently, the *R* configuration to the fast eluting one.

The lowest energy conformation values generated by the Genetic Algorithm search used are -10.55 kcal mol^{-1} for the **se** diastereoisomer (suggested *S* phosphorus configuration) and -6.45 kcal mol^{-1} for the **fe** diastereoisomer (suggested *R* phosphorus configuration).

In summary, a new series of naphthyl phosphoramidates of BVdU has led to a significant improvement in the cytostatic activity of the parent lead compound thymectacin, against a panel of different cancer cell lines. Separation of the diastereoisomeric mixture of compound **10** has shown to be a useful approach to further enhance the anticancer effect of phosphoramidate ProTides.

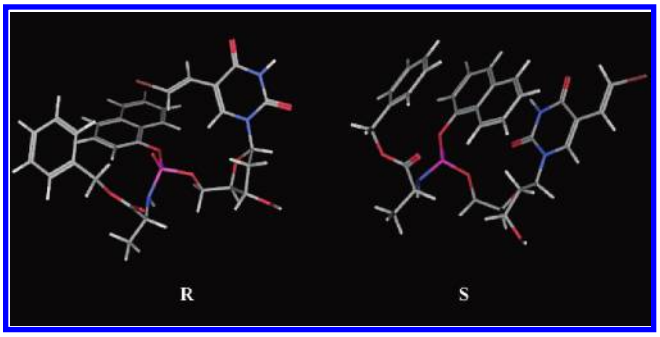


Figure 2. Lowest energy conformations of the two diastereoisomers of compound **10**.

anticancer ProTide for the first time, by a combination of NMR and conformational studies.

Supporting Information Available: Synthesis, NMR, HPLC, low resolution mass, elemental analysis data, and conformational and biological evaluation methods' descriptions. This material is available free of charge via the Internet at http://pubs.acs.org.

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