Stereochemistry of rHint1 hydrolase assisted cleavage of P–N bond in nucleoside 5′-*O*-phosphoramidothioates†

Agnieszka Krakowiak,[a] Renata Kaczmarek,[a] Janina Baraniak,[a] Michał Wieczorek[b] and Wojciech J. Stec*[a]

Received (in Cambridge, UK) 18th October 2006, Accepted 1st February 2007
First published as an Advance Article on the web 22nd February 2007
DOI: 10.1039/b615160d

The Hint-1 hydrolase assisted cleavage of the P–N bond in adenosine-5′-*O*-[*N*-(tryptophanylamide)]phosphoramidothioate proceeds with retention of configuration at the phosphorus atom which is consistent with the formation of a covalent enzyme–substrate complex.

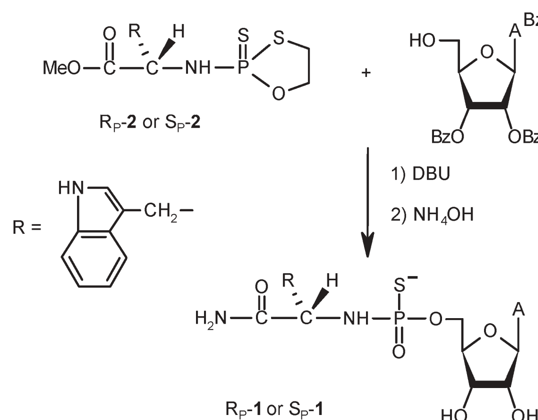
The enhancement of the therapeutic activity of antiviral and anticancer nucleoside analogs and suppression of their side-effects are continuing goals of several research establishments.[1] As demonstrated in numerous studies,[2] therapeutic nucleosides must be converted into their 5′-*O*-triphosphates to achieve biological activity. In order to overcome restrictions imposed by the intracellular phosphorylation mechanism on antiviral and anti-tumor nucleoside analogs, several prodrug approaches have been designed, including phosphoramidate di- and monoesters derived from amino acids.[3] Those classes of nucleoside derivatives have been selected because of the relatively low stability of the P–N bond in cellular media.[4] Although the enzyme responsible for hydrolysis of phosphoramidates has not been established, involvement of putative phosphoramidase in the intracellular release of nucleoside 5′-*O*-phosphate has been demonstrated.[5] Recently, Brenner *et al.* reported that AMP–lysine and AMP–alanine were hydrolyzed by both *Saccharomyces cerevisiae* Hnt1 and rabbit Hint1 hydrolases and suggested that Hint hydrolases might be responsible for nucleoside monophosphoramidate prodrug activation *in vivo*.[6] Because of this proposal, the mechanism of phosphoramidase activity is significant and the possible involvement of a covalent nucleotidyl–enzyme intermediate is an intriguing question.[7] While, in the case of the Fhit protein, the involvement of such an intermediate has been demonstrated by Frey *et al.*,[8] for the Hint enzyme responsible for P–N bond cleavage, which belongs to a different branch of the HIT (histidine triad) family, evidence for a nucleotidyl–enzyme intermediate has not yet been presented. Therefore, stereochemical studies on the Hint assisted P–N bond cleavage are important, since inversion of configuration at the phosphorus atom would exclude a double-displacement mechanism.[7]

[a]*Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, 112 Sienkiewicza Str., 90-363 Łódź, Poland. E-mail: wjstec@bio.cbmm.lodz.pl; Fax: (+48) 42-681-54-83; Tel: (+48) 42-680-32-20*
[b]*Institute of Chemistry and Environmental Protection, Pedagogical University of Częstochowa, AL. Armii Krajowej 13/15, 42-200 Częstochowa, Poland*

† Electronic supplementary information (ESI) available: Experimental details for the synthesis of **1** and **2**, and crystallographic data. See DOI: 10.1039/b615160d

Here, we report on the stereochemistry of the reaction catalyzed by rabbit Hint1, using the P-diastereoisomers of adenosine-5′-*O*-[*N*-(tryptophanylamide)]phosphoramidothioate (**1**). Stereo-controlled synthesis of diastereoisomers of **1** was feasible *via* our oxathiaphospholane approach to the synthesis of P-chiral diesters and amidoesters of phosphoric and phosphorothioic acids.[9] Accordingly, *N*-(2-thiono-1,3,2-oxathiaphospholanyl)tryptophan methyl ester (**2**) was employed as a precursor in the synthesis of phosphoramidothioate **1** (Scheme 1). Compound **2** was obtained as a mixture of two diastereoisomers, in a 1 : 1 ratio, from the reaction of tryptophan methyl ester hydrochloride with 2-chloro-1,3,2-oxathiaphospholane in the presence of elemental sulfur.[10] The isomers were separated by fractional crystallization. The absolute configuration at phosphorus of individual species **2** has been assigned by X-ray analysis‡ (see also the supporting information†); the diastereoisomer absorbing at lower field in the ^{31}P NMR spectrum has been assigned as R_P-**2**. Its P-stereoretentive reaction with $N^6,O^{2'},O^{3'}$-tribenzoyladenosine in the presence of DBU, followed by removal of protective groups, provided S_P-**1** (see the supporting information†). Although both diastereoisomers of **1** served as substrates for the rHint1 enzyme,§ the diastereoisomer R_P-**1** was hydrolyzed some 4 times faster than its counterpart S_P-**1** (Table 1). The hydrolysis of R_P-**1**¶ was performed at pH 7.5 to avoid chemical cleavage of the acid-labile P–N bond and loss of the sulfur from the resulting AMPS.[8a]

Interestingly, although the reaction was terminated after 2 h, the process of P–N bond cleavage was accompanied by a parallel enzymatic reaction of P–S bond cleavage (loss of sulfur) and both AMPS and AMP were isolated on RP-HPLC. In an independent experiment, we have demonstrated that the rate of conversion of



Scheme 1 Synthesis of adenosine-5′-*O*-[*N*-(tryptophanylamide)] phosphoramidothioates (**1**).

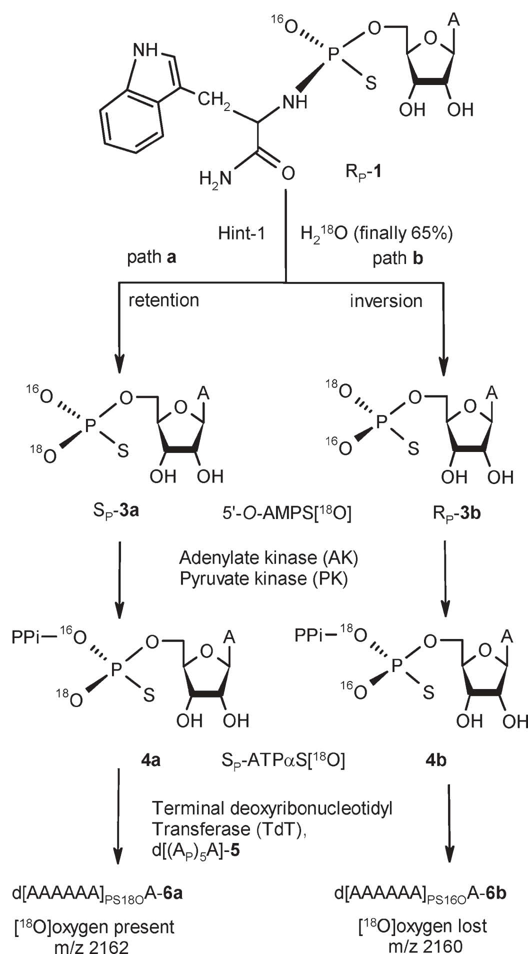
This journal is © The Royal Society of Chemistry 2007

Table 1 Active-site-dependent nucleotide hydrolysis (nmol min^{-1} µg^{-1}) by rabbit Hint and human Fhit

Substrate[a]	Rabbit Hint	Human Fhit	No enzyme
AMPS-Trp-(R_p)-**1**	0.0894 ± 0.0098	—	Not detectable
AMPS-Trp-(S_p)-**1**	0.0241 ± 0.0015	—	Not detectable
AMPS	0.1242 ± 0.0421	0.0046 ± 0.0006	0.0000955 ± 0.0000113

[a] Substrates were assayed at 200 µM and at pH 7.5. Error values (SD) designate the observed ranges for triplicate measurements.

AMPS into AMP is greater than the hydrolysis of the P–N bond in both the isomers (Table 1). These rate constant assignments explain why the predominant product of enzyme-assisted P–N bond cleavage of S_P-**1** is AMP. Under the above conditions, we were able to perform the experiment with rHint1 degradation of R_P-**1** into AMPS-[^{18}O] (**3**) giving sufficient product for stereochemical analysis. Use of the procedure designed in this laboratory (Scheme 2)[11] allowed us to assign the stereochemistry at the phosphorus atom in **3** by a stereospecific two-step conversion into the corresponding ATPαS[^{18}O] (**4**) using tandem adenylate kinase (AK)–pyruvate kinase (PA).|| In this reaction, the [^{18}O]oxygen atom could be located at either the nonbridging (**4a**) or bridging (**4b**) position depending on the stereochemistry of the earlier rHint1 catalyzed hydrolysis. In the next step, after HPLC isolation, ATPαS[^{18}O] was used as the substrate in stereospecific extension of the d[(A_P)$_5$A] primer (**5**) to heptamer **6**, catalyzed by terminal deoxynucleotidyl transferase (TdT). Because of the special conditions used (a concentration of ATPαS equal to that of the primer, and only two hours incubation of the reaction mixture at 37 °C), a heptamer **6** was the main product of this reaction.[11] The heptamer **6b**, resulting from attack on ATPαS[^{18}O] with [^{18}O]oxygen at the bridging position, should not contain an [^{18}O]-label, while heptamer **6a**, formed from ATPαS[^{18}O] with [^{18}O]oxygen at the nonbridging position, should incorporate [^{18}O]. MALDI-TOF MS analysis of the final product **6**** (Fig. 1) unambiguously proved the presence in this heptamer of [^{18}O]oxygen (d[(A_P)$_5$A]$_{PS18O}$A, **6a**). Scheme 2 compares paths **a** and **b**. This means that the overall process involves steps as presented in ***path a*** and proves that rHint1, the enzyme of the first branch of the HIT superfamily, cleaves the P–N bond with stereochemical retention of configuration. This process is consistent with the formation of a covalent enzyme–substrate complex[11]



Scheme 2 Stereochemical consequences of the cleavage of the P–N bond in AMPS-Trp-(R_P)-**1** assisted by Hint-1 hydrolase.

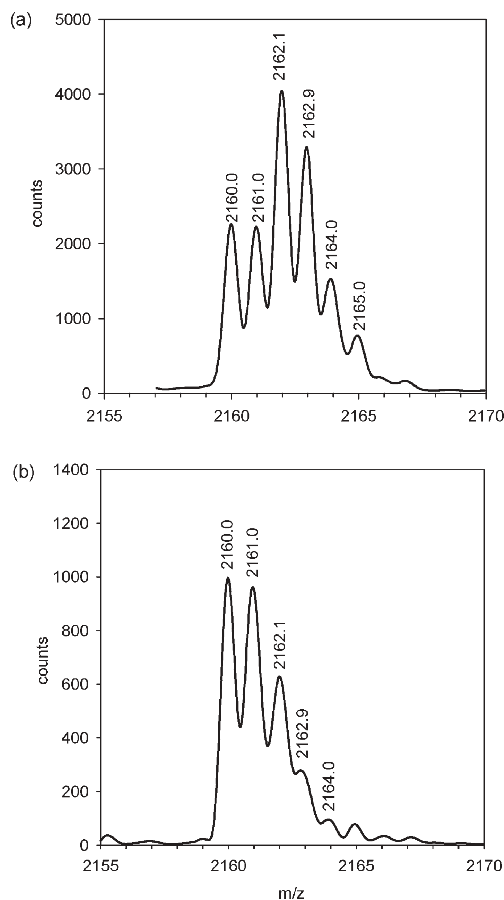


Fig. 1 MALDI-TOF MS analysis of: (a) **6a** obtained from R_P-**1** as described in the text, confirming *path a*; (b) **6b** obtained in the independent experiment of the TdT-assisted reaction of d(A_{p6}A) with commercially available AMPS.

 This journal is © The Royal Society of Chemistry 2007

and is the first report of the stereochemical course of enzymatic P–N bond scission. The result is in agreement with the mechanism of action of two P–O cleaving enzymes belonging to different branches of the HIT superfamily, namely Fhit hydrolase (second branch) and galactose-1-phosphate uridylyltransferase (GalT, third branch).[13,14] The observed loss of sulfur from AMPS as a sequential process accompanying P–N bond cleavage in the action of Hint-1 on nucleoside 5′-*O*-phosphoramidothioates is additionally supported since the AMP isolated contains two [^{18}O]oxygen atoms (MALDI-TOF analysis, Fig. 1). Moreover, preliminary results (not presented here) have shown that this desulfuration process is not ribonucleotide specific. Treatment of 5′-*O*-phosphorothioylated deoxyadenosine, deoxyguanosine, cytidine, and deoxycytidine with Hint-1 effectively converts each of them into the corresponding 5′-*O*-phosphates. Since the Hint enzyme has been shown to have homologs in all forms of life,[14] our observations may become pertinent to the further elucidation of the metabolism of nucleoside 5′-*O*-phosphorothioates, formed as primary products of degradation of antisense oligonucleotide phosphorothioates by 3′-exonucleases. Additional studies concerning the elucidation of the mechanism of the enzyme-catalyzed desulfuration process are in progress.

This project was financially assisted by Polish Ministry of Science and Informatics, (Grants 3T09A05928 (to W. J. S.) and 2PO4A07929 (to A. K.)). The authors are grateful to Dr C. Brenner for providing the Hint plasmid

Notes and references

‡ **2**: $C_{14}H_{17}N_2O_3PS_2$, M = 356.41, monoclinic, space group $P2_1$, a = 8.4537(6), b = 9.5906(7), c = 10.0814(8) Å, β = 104.847(6)°, U = 790.07(10) $Å^3$, T = 110(2) K, Z = 2, μ = 0.451 mm^{-1}, R_{int} = 0.023, $R1$ = 0.0201, w$R2$ = 0.0524 ($I > 2\sigma(I)$). CCDC 614931. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b615160d

§ Wild-type rabbit Hint was expressed and purified in *Escherichia coli* using pSGA02-*HINT* as described in ref. 6.

¶ AMPS-Trp-(R_P)-**1** substrate at a concentration of 1 mM was incubated with homogeneous rabbit Hint enzyme (12 μg) at 30 °C in a 240 μl reaction containing 66 mM sodium/potassium phosphate buffer pH 7.5, 0.5 mM $MgCl_2$ and 80% ^{18}O-enriched water. After 2 h, the reaction was stopped by denaturation at 95 °C (3 min) following chilling in ice. The products were isolated using RP-HPLC (ODS-Hypersil column, 5 μm) with a linear gradient of 0–33% CH_3CN–0.1 M triethylammonium bicarbonate, pH 7.4, 0.85% min^{-1}, at a flow rate of 1 ml min^{-1}. Under these conditions, adenosine 5′-*O*-[^{18}O]-phosphorothioate eluted at 15.50 min. Conversion of AMPS[^{18}O] into ATPαS[^{18}O]-(S_P) and the terminal deoxyribonucleotidyl transferase assay were performed as described in ref. 11.

∥ According to the Cahn–Ingold–Prelog rules, the isotopic replacement $^{16}O \rightarrow {}^{18}O$ in compound **3** changes neither the sense of chirality nor the absolute configuration of the phosphorus atom, as the atomic numbers of the two oxygen isotopes are the same.[12]

** Mass spectrometry analyses were performed as described elsewhere.[11]

1 (*a*) P. Herdewijn, J. Balzarini and E. De Clercq, in *Advances in Antiviral Drug Design*, ed. E. De Clercq, JAI Press, Greenwhich, CN, 1993, vol. 1, pp. 233–318; (*b*) E. De Clercq, *Nat. Rev. Drug Discovery*, 2002, **1**, 13–25.
2 (*a*) J. Balzarini, *Pharm. World Sci.*, 1994, **16**, 113; (*b*) G. S. Ahluwalia, W.-Y. Gao, H. Mitsuya and D. G. Johns, *Mol. Pharmacol.*, 1996, **50**, 160–165; (*c*) P. A. Furman, J. A. Fyfe, M. H. St. Clair, K. J. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrmann, D. P. Bolognesi, S. Broder, H. Mitsuya and D. W. Barry, *Proc. Natl. Acad. Sci. U. S. A.*, 1986, **83**, 8333–8337.
3 (*a*) C. Meier, *Synlett*, 1998, 233–242; C. R. Wagner, V. V. Iyer and E. J. McIntee, *Med. Res. Rev.*, 2000, **20**, 417–451; (*b*) J. Zemlicka, *Biochim. Biophys. Acta*, 2002, **1587**, 276–286; (*c*) D. Cahard, C. McGuigan and J. Balzarini, *Mini-Rev. Med. Chem.*, 2004, **4**, 371–382.
4 T. W. Abraham, T. I. Kalman, E. J. McIntee and C. R. Wagner, *J. Med. Chem.*, 1996, **39**, 4569–4575.
5 (*a*) M. E. Holzer, K. D. Johnson and R. A. Smith, *Biochim. Biophys. Acta*, 1966, **122**, 232–243; (*b*) R. K. Ledneva, N. N. Preobrazhenskaya, N. G. Shinskii, Z. A. Shabarova and M. A. Prokofev, *Dokl. Akad. Nauk SSSR*, 1970, **193**, 1308–1310; (*c*) M. Kuba, T. Okizaki, H. Ohmori and A. Kumon, *Int. J. Biochem.*, 1994, **26**, 235–245; (*d*) E. J. McIntee, R. P. Remmel, R. F. Schinazi, T. W. Abraham and C. R. Wagner, *J. Med. Chem.*, 1997, **40**, 3323–3331.
6 P. Bieganowski, P. N. Garrison, S. C. Hodawadekar, G. Faye, L. D. Barnes and C. Brenner, *J. Biol. Chem.*, 2002, **277**, 10852–10860.
7 P. A. Frey, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1989, **62**, 119–201.
8 (*a*) A. Abend, P. N. Garrison, L. D. Barnes and P. A. Frey, *Biochemistry*, 1999, **38**, 3668–3676; (*b*) K. Huang, A. Arabshahi, Y. Wei and P. A. Frey, *Biochemistry*, 2004, **43**, 7637–7642.
9 W. J. Stec, B. Karwowski, M. Boczkowska, P. Guga, M. Koziołkiewicz, M. Sochacki, M. W. Wieczorek and J. Błaszczyk, *J. Am. Chem. Soc.*, 1998, **120**, 7156–7167.
10 J. Baraniak, R. Kaczmarek, D. Korczyński and E. Wasilewska, *J. Org. Chem.*, 2002, **67**, 7267–7274.
11 (*a*) M. Koziołkiewicz, A. Owczarek, M. Wójcik, K. Domański, P. Guga and W. J. Stec, *J. Am. Chem. Soc.*, 2002, **124**, 4623–4627; (*b*) A. Krakowiak, A. Owczarek, M. Koziołkiewicz and W. J. Stec, *ChemBioChem*, 2002, **2**, 101–109.
12 (*a*) P. A. Frey, in *The Enzymes*, ed. D. S. Sigman, Academic Press, San Diego, CA, 1992, vol. 20, p. 145; (*b*) P. Guga, K. Domański and W. J. Stec, *Angew. Chem., Int. Ed.*, 2001, **40**, 610–613.
13 P. A. Frey, L. J. Wong, K.-F. R. Sheu and S. L. Yang, *Methods Enzymol.*, 1982, **87**, 20–36.
14 C. Brenner, *Biochemistry*, 2002, **41**, 9003–9014.