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PHENYLACETYL GROUP AS ENZYME-CLEAVABLE AMINOPROTECTION OF PURINE NUCLEOSIDES

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Abstract: *N^6-Phenylacetyl-2'-deoxyadenosine and N^2-Phenylacetyl-2'-deoxyguanosine are readily deprotected in reactions catalyzed by free and immobilized penicillin amidase at pH 7.8 and 25° C.*

Despite the relatively weak reactivity of the exocyclic amino group of 2'-deoxyadenosine (dAdo) or 2'-deoxyguanosine (dGuo) under the conditions for nucleotide bond formation, its selective protection is an unavoidable step in oligonucleotide synthesis.[1,2] These 2'-deoxynucleosides can loose their purine residue in either strongly basic or moderately acidic conditions of solid phase synthesis thus generating truncated sequences. The rate of these side reactions is even enhanced when an aroyl group is used for protection of the purine amino function.[3] The effect is reduced using a sterically crowded amidine protection.[4] Furthermore, the growing interest in the application of modified oligonucleotides as gene-suppressing therapeutic reagents[5] requires protecting groups compatible with the intrinsic liability of specific modifications at phosphorous. The use of N^2-phenylacetyl-5'-protected-2'-deoxyguanosine derivatives in oligonucleotide synthesis has already been considered as practical due to their increased solubility in organic solvents.[6]

Enzymic manipulation of protecting groups offers a viable alternative to classical chemical methods.[7,8] *This strategy has never been used in nucleotide synthesis.* Many enzymes work in neutral solutions combining high reaction selectivity with a broad substrate specificity. Thus penicillin amidase (PA) from *E. coli* (E.C. 3.5.1.11.) catalyzes phenylacetyl moiety transfer from/to nucleophiles varying from 6-aminopenicillanic acid to peptide amines.[9-11] Here we report that the phenylacetyl group can be successfully used as an enzyme (PA)-cleavable aminoprotecting group of 2'-deoxyadenosine and 2'-deoxyguanosine.

Phenylacetyl protection of the exocyclic amino groups of the purine nucleosides dAdo and dGuo was performed using trimethylsilylation of 3'- and 5'-OH functions as transient protection by a specific adaptation[12] of the procedure originally designed for dGuo.[6] The yields were 80% for dAdo and 75% for dGuo derivative. The crystalline protected nucleosides N^6-Ac(Ph)dAdo and N^2-Ac(Ph)dGuo were characterized by t.l.c., HPLC, IR, and ^{1}H NMR.[13]

N^6-Ac(Ph)dAdo and N^2-Ac(Ph)dGuo were subjected to deprotection, catalyzed by free or immobilized penicillin amidase[14] (Scheme). To a 0.125 mM solution of the corresponding nucleoside derivative in phosphate buffer pH 7.8 (I=0.2) was added free (3.5×10^{-7}M) or immobilized (7.5×10^{-7}M) enzyme. The reaction mixture was incubated

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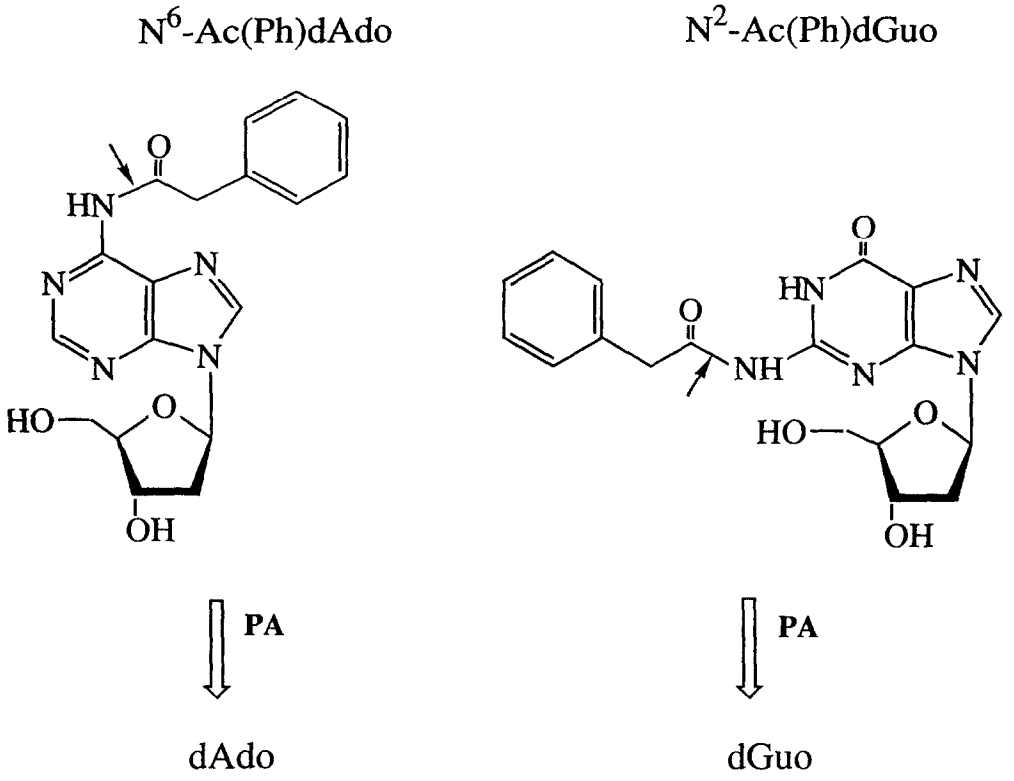

(or stirred in the case of immobilized enzyme) at room temperature and analyzed by HPLC. Complete deprotection was observed, the deprotection time depending on the nature of the nucleoside or the biocatalyst (Table).

The kinetics of the penicillin amidase-catalyzed deprotection was studied under the same conditions with substrate concentrations ranging from 0.1×10^{-3} to 1×10^{-3} M. The reaction was followed by HPLC. The catalytic parameters were derived from the initial rate of phenylacetic acid formation using the computer program Enzfitter[15] for nonlinear regression analysis. The calculated values and the data obtained previously for penicillin G, phenylacetyl-glycine and phenylacetyl-aspartame are summarized in the Table.

Due to the leaving group specificity of penicillin amidase, the reactivity (k_{cat}/K_M - values) of N-phenylacetyl-purine nucleosides is lower than that of its natural substrate N^6-phenylacetyl penicillanic acid (Penicillin G). The enzyme leaving group binding subsite, however, tolerates purine nucleoside residues in the transition state (similar order of magnitude k_{cat}-values) and binds weakly their ground state (three orders of magnitude higher K_M-values). These data suggest that penicillin amidase would be effective in multiple deprotection of oligodeoxynucleotides containing N-phenylacetylated purine nucleobases under mild conditions. The acceptable deprotection times (Table) support this conclusion. Comparison of the kinetic parameters in the Table allows

Table. Kinetic Parameters for Penicillin Amidase-catalyzed Deprotection of N-Phenylacetylated Purine Nucleosides and Related Compounds at pH 7.8 (I=0.2) and 25° C. S.D. less than 10%.

SUBSTRATES	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $s^{-1}\mu M^{-1}$	Deprotection Time[a], min	
				Free PA	Immobilized PA
N^6-Ac(Ph)-dAdo	2	460	0.004	30	120
N^2-Ac(Ph)-dGuo	18	800	0.023	15	60
Ac(Ph)-Aspartame[b]	35	670	0.052	-	150[c]
Ac(Ph)-Gly-OH	47	5.2	9.0[d]	-	25
N^6-Ac(Ph)-APA[e]	50	10	10.0	-	25

[a] 0.125 mM substrate, 3×10^{-7}M free PA or 7.5×10^{-7}M immobolized PA; [b] Ref. 11; [c] 200 mM substrate, 3×10^{-3}M immobilized PA; [d] Ref. 16; [e] Penicillin G

us to infer that treatment of N^4-phenylacetyl-2'-deoxycytidine (when available) with penicillin amidase should also result in the cleavage of the protecting group.

Therapeutic intervention on the level of the nucleic acids requires synthesis of modified oligonucleotides on a large scale.[5] This development calls for enhanced protecting group techniques not used before in gene synthesis, where tiny quantities are enough for molecular cloning. Enzymic deprotection techniques are potentially an integral part of this new strategy.

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12. The nucleoside (4 mmol) was dried and suspended in dry pyridine (20 ml). Trimethylchlorosilane was added and the mixture was stirred at ambient temperature until silylation was complete (t.l.c.). Phenylacetyl chloride (6mmol) was mixed with 1-hydroxybenzotriazole (6.4 mmol) and suspended in dry acetonitrile (2ml).

Pyridine (2 ml) was added to dissolve any precipitated salt. This colorless solution was added dropwise at 0° C to the reaction mixture containing the silylated nucleoside. The reaction mixture was stirred overnight at ambient temperature. The mixture was cooled to 0°C and the reaction was stopped by addition of water (5 ml). After 5 min at 0°C, 25% aqueous ammonia was added. The aminolysis of the silyl ethers was completed in 30 min (t.l.c.). The solvents were removed and the residue was dissolved in water and extracted several times with ethyl acetate. The aqueous phases were evaporated until the product crystalized. The crystals were filtered , washed with water, and dried.

13. The ^{1}H NMR spectra were recorded on a Bruker 250 spectrometer at 250 MHz in DMSO with TMS as internal standard. IR spectra were recorded on a Bruker IFS 113 V spectrometer. HPLC analyses were performed on a LKB chromatography system consisting of solvent delivery system, gradient controller, UV–VIS detector and using Merck RP-18 (5 μM) column (isocratic elution with 30% (v/v) MeOH and 70% (v/v) 0.067 M KH_2PO_4; detection at 225 nm). Thin layer chromatography was carried out on silica sheets (Kiselgel 60), elution with $CHCl_3$ / MeOH (9:1).
N^2-Ac(Ph)dGuo: t.l.c.: R_f=0.50; ^{1}H NMR (DMSO-d_6, ppm): δ 3.50 (m, -CH_2 Ac(Ph), 2H), δ 6.13 (dd, 1´ H, 1H), δ 6.60 (t, -NHCO, J=6.9 Hz, 1H), δ 7.26 (m, Ph Ac(Ph) , 5H); IR (KBr, cm^{-1}) ν: 3403 (NH), 1711, 1680, 1571 (C=O, C=N, C=C); HPLC: R_t = 2´50´´.
N^6-Ac(Ph)dAdo: t.l.c.: R_f=0.54; ^{1}H NMR (DMSO-d_6, ppm): δ 3.46 (m, -CH_2 Ac(Ph), 2H), δ 6.44 (dd, 1´H, 1H), δ 7.25 (t, -NHCO, J=6.5 Hz, 1H), δ 7.34 (m, Ph Ac(Ph), 5H); IR (KBr, cm^{-1}) ν: 3440 (NH), 1731, 1690, 1587, (C=O, C=N, C=C); HPLC: R_t=3´.

14. The free penicillin amidase was purified from a crude enzyme preparation (Röhm Pharma) by affinity chromatography on immobilized monoclonal antibodies.[17] The immobilized penicillin amidase was from Sclava The enzyme concentrations of the free and immobilized enzyme were determined by active site titration with phenylmethylsulphonyl fluoride[18] using 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) as substrate.

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