Efficient Synthesis of Gemcitabine 5′-*O*-Triphosphate Using Gemcitabine 5′-*O*-Phosphoramidate as an Intermediate

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Received: 15.04.2014; Accepted after revision: 28.05.2014

Abstract: A new efficient approach for the synthesis of gemcitabine triphosphate has been developed. The method is based on the ring-opening reaction of 2-cyanoethoxy-2-oxo-1,3,2-oxathiaphospholane with protected gemcitabine in the presence of DBU. Subsequent treatment of gemcitabine monophosphate with DCC in the presence of ammonia provides gemcitabine 5′-*O*-phosphoramidate. Finally, this compound, on reaction with pyrophosphate, furnishes gemcitabine 5′-triphosphate in 50% yield.

Key words: oxathiaphosphorylation, gemcitabine 5′-*O*-phosphate, gemcitabine 5′-*O*-phosphoramidate, gemcitabine 5′-*O*-triphosphate

Gemcitabine (difluorodeoxycytidine, dFdC, Gemzar®) is a deoxycytidine analogue with two fluorine atoms substituted for the two hydrogen atoms in the 2′-position of the deoxyribose moiety. dFdC is registered as an anticancer drug for the treatment of a number of different solid tumors including nonsmall cell lung (NSCL), pancreatic, ovary, bladder, and breast cancer.[1–3] Gemcitabine is also active against lymphomas.[4] Studies on the metabolism of gemcitabine have demonstrated that this compound is a prodrug, which is subsequently converted intracellularly by deoxycytidine kinases into the corresponding 5′-monophosphate (dFdCMP), 5′-diphosphate (dFdCDP), and, finally, to its active 5′-triphosphate form (dFdCTP).[5] These metabolites inhibit two cellular processes required for DNA biosynthesis. dFdCDP is a very potent inhibitor of ribonucleotide reductase[6] (RNR), while dFdCTP, after being incorporated into the C sites of the DNA during replication, inhibits DNA polymerases.[6,7] Furthermore, 2′,2′-difluoro-2′-deoxycytidine triphosphate is also inserted into RNA, at a similar level as in DNA.[8]

Compared with ara-C, gemcitabine is phosphorylated more efficiently and is eliminated more slowly, thus offers a longer retention time of the active forms in tumor cells.[9] Moreover, gemcitabine cytotoxicity is enhanced by a number of unique self-potentiating mechanisms that maintain high intracellular concentrations of the active metabolites. It is worth mentioning that dFdCTP is used as the standard in imaging studies using a radiolabeled probe for assessing the uptake and retention time of gemcitabine in tumors and potentially identifying tumors sensitive to the drug.[10]

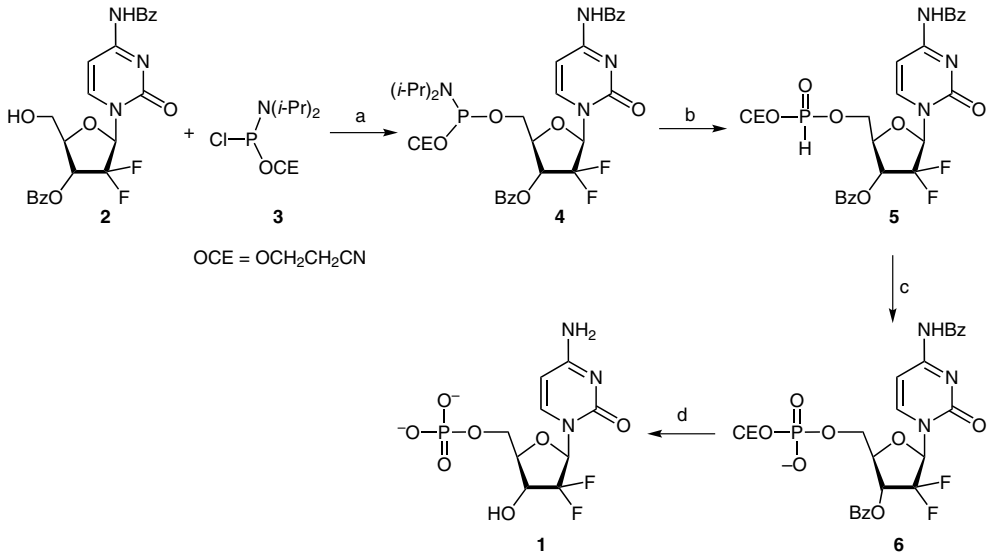
Only one synthetic method for the preparation of gemcitabine 5′-*O*-triphosphate has been reported in the literature, in which activated gemcitabine 5′-*O*-phosphate was used as a key intermediate.[11] This intermediate was obtained by phosphorylation of the 5′-hydroxyl function of gemcitabine with $POCl_3$ in trimethyl phosphate, followed by transformation to a 1-methylimidazolium derivative. Further treatment with an acetonitrile solution of tris(tetrabutylammonium)hydrogen pyrophosphate gave dFdCTP in 17% yield.

Due to our long-term interest in the synthesis of nucleoside phosphates and polyphosphates[12] we wished to elaborate a procedure for the selective preparation of gemcitabine monophosphate and its further transformation into gemcitabine triphosphate.

Our first attempt towards the synthesis of gemcitabine 5′-*O*-phosphate (**1**) was based on phosphoramidite chemistry, which was also used by Desmaële for the synthesis of squalenoyl gemcitabine monophosphate.[13] N^4,$O^{3'}$-dibenzoylgemcitabine (**2**) was phosphitylated by means of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (**3**, Scheme 1) to yield **4**, which, after hydrolysis in the presence of 1-*H*-tetrazole, gave the expected gemcytabine *H*-phosphonate derivative **5** isolated by silica gel column chromatography in 54% yield and characterized by ^{31}P NMR [δ (CD_3CN) = 9.33 and 8.74 ppm] and MS–FAB {*m/z* = 587 [M – 1]}. Compound **5** was oxidized with an iodine–base–water mixture to yield **6**, which was then debenzoylated with concentrated aqueous ammonia. Unfortunately, the desired gemcitabine 5′-*O*-phosphate (**1**) was isolated by ion-exchange column chromatography in only 3% yield (characterized by ^{31}P NMR and MS–FAB). One might presume that the retro-Michael deprotection of the cyanoethyl group occurring under ammonia treatment is responsible for this extremely low yield.

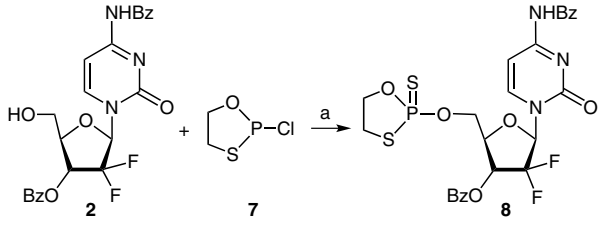
However, ^{31}P NMR inspection of the reaction mixture obtained after oxidizing of **5** showed, alongside the signal corresponding to **6** (δ = –1.32 ppm; ca. 20%), unidentified signals at δ = 14.80 and 11.77 ppm.

SYNLETT 2014, 25, 1851–1854
Advanced online publication: 09.07.2014
DOI: 10.1055/s-0034-1378353; Art ID: st-2014-d0319-l



Scheme 1 Synthetic approach to the gemcitabine 5′-*O*-phosphate (**1**) based on phosphoramidite-type of chemistry. *Reagents and conditions*: (a) DIPEA, CH_2Cl_2; (b) 1-*H*-tetrazole, MeCN–H_2O; (c) I_2–H_2O–pyridine, Et_3N; (d) NH_4OH.

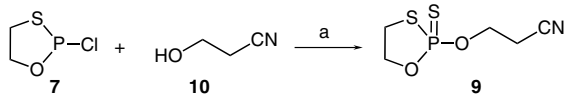
In the light of the above results we turned our attention to the oxathiaphospholane methodology, originally developed by Stec for the stereocontrolled synthesis of P-chiral analogues of oligonucleotides.[14] This methodology has been also used for thiophosphorylation and phosphorylation of various alcohols, including nucleosides.[12a,15] In this approach, 5′-*O*-(2-thiono-1,3,2-oxathiaphospholane) derivatives of nucleosides, on reacting with 3-hydroxypropionitrile in the presence of DBU, were transformed into the corresponding nucleoside-5′-*O*-(*O*-2-cyanoethyl-phosphorothioate)s. After removing the 2-cyanoethyl moiety and benzoyl protecting groups with concentrated aqueous ammonia, the desired phosphorothioate monoesters were isolated in good to reasonable yields. The phosphorothioate compounds could then be oxidized to the corresponding phosphates upon treatment with $PhIO_2$.[16]



Scheme 2 Oxathiaphosphorylation of the protected gemcitabine (**2**). *Reagents and conditions*: (a) S_8, pyridine.

Unfortunately, direct oxathiaphosphorylation of the protected gemcitabine (**2**) by means of 2-chloro-1,3,2-oxathiaphospholane (**7**) in the presence of elemental sulfur gave the expected compound **8** in only 15% yield (Scheme 2). Hence, 2-cyanoethoxy-2-thiono-1,3,2-oxathiaphospholane (**9**) was synthesized as an alternative phosphorothioylating reagent.[17]

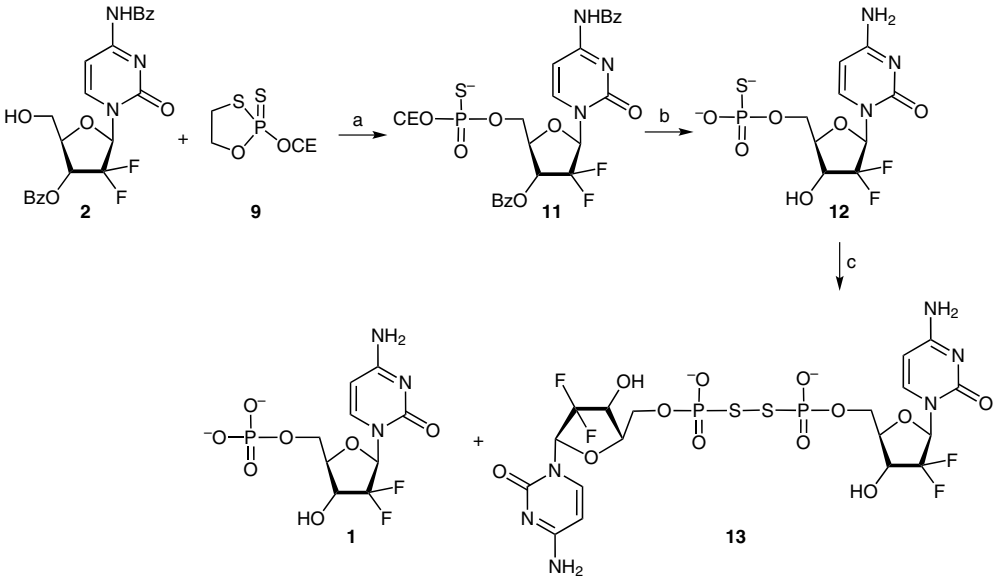
To obtain this reagent 3-hydroxypropionitrile (**10**) was reacted with 2-chloro-1,3,2-oxathiaphospholane (**7**)[18] in the presence of elemental sulfur in pyridine (Scheme 3).



Scheme 3 Synthesis of 2-cyanoethoxy-2-thiono-1,3,2-oxathiaphospholane (**9**). *Reagents and conditions*: (a) S_8, pyridine.

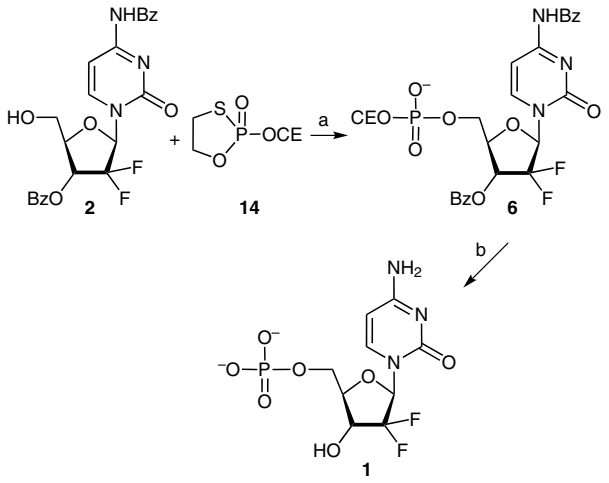
The reaction of crude **9** with protected gemcitabine (**2**) was carried out in the presence of DBU in acetonitrile (Scheme 4). The diester **11** so obtained was converted into the desired phosphorothioate **12** by overnight treatment with concentrated aqueous ammonia at room temperature, and **12** was subsequently isolated from the reaction mixture by means of ion-exchange chromatography on DEAE-Sephadex A-25 in 67% yield. Its structure was confirmed by ^{31}P NMR spectroscopy [δ (D_2O) = 43.90 and 43.55 ppm] and MS–FAB {*m/z* = 358 [M – 1]} analyses. Unexpectedly, compound **12** upon treatment with $PhIO_2$ furnished mainly (40% yield) a gemcitabine disulfide derivative **13** {^{31}P NMR: δ (D_2O) = 16.80 ppm; MS–FAB: *m/z* = 715 [M – 1]} while desired **1** was obtained in only 2% yield.

To avoid the use of an oxidizing reagent with **12** we modified our approach and oxidized **9** with selenium dioxide in acetonitrile.[15c] The ^{31}P NMR spectrum of the reaction mixture recorded after ten minutes revealed a signal at around δ = 45 ppm, indicating the formation of 2-cyanoethoxy-2-oxo-1,3,2-oxathiaphospholane (**14**), which was reacted with protected gemcitabine (**2**) for four hours in the presence of DBU in acetonitrile (Scheme 5). In this case the desired gemcitabine 5′-*O*-phosphate (**1**) was



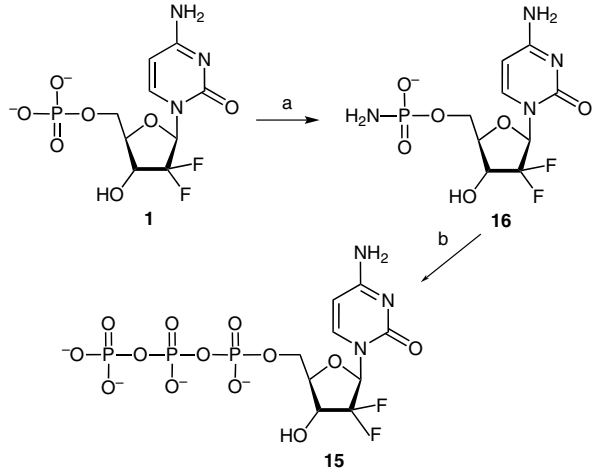
Scheme 4 Reaction of protected gemcitabine (**2**) with 2-cyanoethoxy-2-thiono-1,3,2-oxathiaphospholane (**9**). *Reagents and conditions*: (a) DBU, MeCN; (b) NH_4OH; (c) $PhIO_2$, H_2O.

formed in 67% overall yield. Its structure was confirmed by ^{31}P NMR spectroscopy [δ (D_2O) = 3.69 ppm] and MS–FAB analysis {*m/z* = 342 [M – 1]}.[19]



Scheme 5 Synthetic approach to the gemcitabine 5′-*O*-phosphate (**1**) based on using 2-cyanoethoxy-2-oxo-1,3,2-oxathiaphospholane as a phosphorylating reagent. *Reagents and conditions*: (a) DBU, MeCN; (b) NH_4OH.

In the next step gemcitabine 5′-*O*-phosphate (**1**) was converted into gemcitabine 5′-*O*-triphosphate (**15**). The most widely used method of synthesis of nucleoside 5′-*O*-triphosphates involves a nucleophilic attack of a pyrophosphate anion on a DCC/*N*-activated nucleoside 5′-*O*-phosphate.[20] We took advantage of this type of activation and activated gemcitabine 5′-monophosphate (**1**) with DCC/ammonium hydroxide in DMF (Scheme 6). After heating at 80 °C for eight hours, gemcitabine 5′-*O*-phosphoramidate (**16**) was formed and isolated in 79% yield using ion-exchange chromatography on DEAE-Sephadex A-25. Its structure was confirmed by ^{31}P NMR spectroscopy [δ (D_2O) = 9.41 ppm] and MS–FAB analysis {*m/z* = 341 [M – 1]}.[21]



Scheme 6 Synthesis of gemcitabine 5′-*O*-triphosphate (**15**) using gemcitabine 5′-*O*-phosphoramidate (**16**) as an intermediate. *Reagents and conditions*: (a) DCC, NH_4OH; (b) bis-(tri-*n*-butyl)ammonium pyrophosphate (**17**), DMF.

Finally, **16** was converted into **15** using the procedure employed by Tomasz for the preparation of 5′-*O*-triphosphate derivatives of 3′,5′-diribonucleoside phosphates.[22] The reaction was carried out in the presence of bis-(tri-*n*-butyl)ammonium pyrophosphate (**17**, Scheme 6) in DMF solution at 65 °C for 13 hours and its progress was monitored by ^{31}P NMR. Gemcitabine 5′-*O*-triphosphate (**15**, dFdCTP) was isolated as its tris(triethylammonium) salt in 50% yield using HPLC and was characterized by ^{31}P

NMR spectroscopy [δ (D_2O) = –10.43 (d), –11.04 (d), and –22.59 (t) ppm] and MS analysis {MALDI, *m/z* = 501.7 [M – 1]}.[23]

In conclusion, a new efficient route for the synthesis of gemcitabine 5′-*O*-triphosphate (dFdCTP) has been developed using gemcitabine monophosphate (**1**) as an intermediate. Whereas some strategies presented herein have been used previously for the synthesis of diverse nucleoside monophosphates,[12a,15,16b] they could not be implemented for the preparation of the desired compound **1**. dFdCMP (**1**) was eventually prepared by the ring-opening reaction of 2-cyanoethoxy-2-oxo-1,3,2-oxathiaphospholane (**14**) with protected gemcitabine (**2**) in the presence of DBU. Subsequent treatment of **1** with DCC in the presence of ammonia provided gemcitabine 5′-*O*-phosphoramidate (**16**) in good yield, and this activated form of monophosphate was reacted with the pyrophosphate anion furnishing gemcitabine 5′-*O*-triphosphate (**15**) in reasonable yield.

Acknowledgment

These studies were supported financially by the Statutory Funds of CMMS PAS.

Supporting Information for this article, including detailed experimental procedures for the syntheses of compounds **1**, **2**, **9**, **14**, **15** and **16** is available online at http://www.thieme-connect.com/products/ejournals/journal/10.1055/s-00000083.

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(19) **Experimental Procedure for the Synthesis of Compound 1**
To a mixture of compound **2** and DBU in MeCN, crude oxathiaphospholane **14** was added. After stirring at r.t. for 4 h the mixture was evaporated in vacuo, and the residue was dissolved in 20% aq NH_3 and left for 24 h at r.t. The mixture was then evaporated, and product **1** was isolated by ion-exchange chromatography in 67% yield.
^{31}P NMR (202.45 MHz, D_2O): δ = 3.69 ppm. ^1H NMR (500 MHz, D_2O): δ = 7.84–7.79 (d, 1 H), 6.17–6.12 (t, 1 H), 6.03–6.00 (d, 1 H), 4.41–4.33 (m, 1 H), 4.17–4.11 (m, 1 H), 4.10–4.05 (m, 1 H), 4.03–3.98 (m, 1 H) ppm. ESI-MS [M – 1]: *m/z* = 342.

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(21) **Experimental Procedure for the Synthesis of Compound 16**
Compound **1** was dissolved in a mixture of 2 M NH_4OH and formamide, and to this solution DCC dissolved in *t*-BuOH was added. The reaction mixture was heated at 80 °C for 10 h and then allowed to stand overnight at r.t. The mixture was evaporated in vacuo, and the product was isolated by ion-exchange chromatography in 78% yield.
^{31}P NMR (202.45 MHz, D_2O): δ = 9.41 ppm. ^1H NMR (500 MHz, D_2O): δ = 7.80–7.76 (d, 1 H), 6.19–6.13 (t, 1 H), 6.08–6.02 (d, 1 H), 4.41–4.37 (m, 1 H), 4.14–4.10 (m, 1 H), 4.08–4.01 (m, 1 H), 4.00–3.96 (m, 1 H) ppm. ESI-MS [M – 1]: *m/z* = 341.

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(23) (a) **Experimental Procedure for the Synthesis of Compound 15**
Compound **16** was dissolved in anhydrous DMF, and bis-(tri-*n*-butyl)ammonium pyrophosphate (**17**) in DMF was added. After heating the homogeneous solution in a stoppered flask at 65 °C for 13 h the mixture was evaporated, and the product was isolated using HPLC in 50% yield.
^{31}P NMR (202.45 MHz, D_2O): δ = –10.43 (d), –11.04 (d), –22.59 (t) ppm. ^1H NMR (500 MHz, D_2O): δ = 7.83–7.80 (d, 1 H), 6.14–6.09 (t, 1 H), 6.08–6.05 (d, 1 H), 4.46–4.38 (m, 1 H), 4.29–4.23 (m, 1 H), 4.18–4.13 (m, 1 H), 4.08–4.04 (m, 1 H) ppm. MALDI-MS [M – 1]: *m/z* = 501.7.

