A Universal Biocatalyst for the Preparation of Base- and Sugar-Modified Nucleosides *via* an Enzymatic Transglycosylation

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Dedicated to Prof. Dr. *Wolfgang Pfleiderer* on the occasion of his 75th birthday

The *E. coli* BMT-4D/1A cells have been selected according to *Munch-Petersen et al.* They carry two regulatory mutations (cytR and deoR) and are able to synthesize constitutively nucleoside-catabolizing enzymes, *e.g.*, cells that possess high UPase and PNPase activities. The cells have been cross-linked by glutaraldehyde to afford a biocatalyst that retained high UPase and PNPase activities and was comfortable for repeated use. An incubation of 2′-deoxyguanosine (**1**) and 2-chloroadenine (**2**) (molar ratio 3:1) in K-phosphate buffer (10 mM; pH 7.0) in the presence of the biocatalyst at 65° for 7 h resulted in quantitative transformation of **2** into 2-chloro-2′-deoxyadenosine (**4**; *cladribine*) that was isolated in 81% yield (*Scheme 1*). Similarly, the reaction of guanosine (**5**) and 1,2,4-triazole-3-carboxamide (**6**) (molar ratio 1:1) in K-phosphate buffer (10 mM; pH 7.0) in the presence of the biocatalyst at 60° for 30 h led to the formation of 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide (**8**; *ribavirin*) in 90–92% yield (67–70% isolated yield) (*Scheme 2*).

Introduction. – Nucleoside analogues are a mainstream in the treatment of many viral infections and play a significant role in the chemotherapy of different malignancies [1–4]. Most of them are produced by chemical methods employing the natural nucleosides as starting compounds, or by a convergent approach *via* the condensation of the carbohydrate precursor and heterocyclic base. The latter strategy suffers from many drawbacks, most serious of which are the low stereospecificity of the glycosidic-bond formation and ambiguous regiospecificity, especially in the case of purine bases. As a result, chemical schemes of nucleoside-analogue preparation are rather complicated and accompanied by a tedious isolation of the individual desired compounds [5].

Considerable progress in the preparation of nucleoside analogues under consideration was achieved by combination of chemical methods and biochemical transformations (for reviews, see [6]). The enzymatic equilibrium-transfer reaction of the sugar moiety of one nucleoside to the heterocyclic base was discovered by *Krenitsky* in the 60's [7][8]. It was established that this equilibrium reaction is catalyzed by purine nucleoside phosphorylase (PNPase) in the case of purine nucleosides and purine bases, and by uridine (thymidine) phosphorylases (UPase or TPase) in the case of pyrimidine nucleosides and pyrimidines. More than a decade later, it was shown that this reaction can be used for the preparation of base- and sugar-modified nucleosides [9–13].

Employment of enzymes or the whole cells as biocatalysts results in a principal improvement of key stages of the preparation of nucleoside analogues, *viz.*, allowing fulfillment of the stereo- and regioselective formation of the glycosidic bond. From the published enzymatic nucleoside syntheses, it becomes obvious that the application of chemical-enzymatic methodology for the preparation of nucleosides of medicinal importance may essentially contribute to an improvement of cost-effectiveness ratios *vs.* current chemical processes. At the same time, despite the widespread appreciation of benefits of 'chemical biotechnology', a number of problems should be overcome in order to displace synthetic methods, which have, in some cases, a highly established position. Biocatalysts should possess higher enzymatic efficiency and operational stability, and need to be more cost-effective.

Two approaches have successfully been employed for the pentofuranosyl-transfer reaction catalyzed by nucleoside phosphorylases: one of these makes use of the purified enzymes, the other is based on the use of the whole bacterial cells displaying the UPase and/or TPase, and PNPase activities as a biocatalyst [6]. During the course of our studies directed towards the search of bacterial cells manifesting the high UPase or/and TPase and PNPase activities, we have selected three strains of *E. coli*. The first of them, *E. coli* BM-11, displayed a rather broad specificity in relation to the bases and sugar-modified nucleosides [14] and was successfully employed for the synthesis of a variety of base- and sugar-modified nucleosides [15–19]. It was found that the utilization of cells cross-linked by glutaraldehyde (GA) offers some experimental advantages over unmodified *E. coli* BM-11 cells [17–19]. Furthermore, the *E. coli* BM-11 cells display, besides the high UPase or/and TPase and PNPase activities, high cytidine deaminase (CDase) activity that enables the use of cytosine nucleosides as donors of pentofuranose moiety in the enzymatic transglycosylation. These findings have been successfully used in the synthesis of 9-(β-D-arabinofuranosyl)guanine (ara-G), employing 1-(β-D-arabinofuranosyl)cytosine (ara-C) as a donor of the arabinofuranose residue and guanosine (Guo) or 2′-deoxyguanosine (dGuo) as depot forms of guanine *via* a cascade of enzymatic reactions [18]. The second strain, *E. coli* BMT-1D/1A, proved to be somewhat more efficient in the synthesis of *a*) thymidine, using either *i*) dGuo, 2′-deoxycytidine (dCyd), or 2′-deoxyadenosine (dAdo), or *ii*) a mixture of the same 2′-deoxynucleosides resulting from an enzymatic hydrolysis of DNA as donors of 2-deoxyribofuranose moiety [20], *b*) dAdo [21], *c*) a panel of ribo- and 2-deoxyribofuranosides of 1-deaza- and 3-deazapurines [22] and benzimidazoles [22][23], and *d*) 2-chloro-2′-deoxyadenosine (*cladribine*) [24]. Finally, a slightly modified strain, *E. coli* BMT-4D/1A, was found to be essentially more efficient *vs.* the parent *E. coli* BMT-1D/1A in the synthesis of 2′-deoxy-2′-fluoroguanosine [25] and 2,6-diamino-9-(3-deoxy-β-D-*erythro*-pentofuranosyl)purine [26].

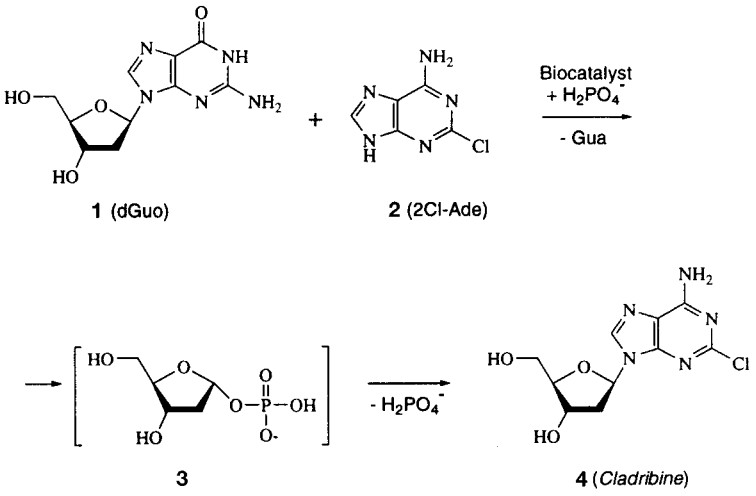
Results and Discussion. – The present study was undertaken in order to develop a practical synthesis of 2-chloro-2′-deoxyadenosine (**4**) and 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide (**8**; *ribavirin*), using dGuo or Guo as donors in the enzymatic transglycosylation of 2-chloroadenine (**2**; 2Cl-Ade) and 1,2,4-triazole-3-carboxamide (**6**; TCA), respectively. A choice of these analogues is undoubtedly evoked by their medicinal importance (see, *e.g.*, the leading references [27] and [28], resp.). The *E. coli* BMT-4D/1A cells have been cross-linked by glutaraldehyde (GA-cells) to afford a

biocatalyst that retained high UPase and PNPase activities and was suitable for repeated use. The cross-linking of the cells by GA seems to be advantageous over the alginate gel-entrapped cells used by *Holy* and co-workers [29] for a related biocatalyst because the GA cells retain operational stability and catalytic activity up to 65° in phosphate buffer. Moreover, the GA cells have been shown to be more efficient *vs.* unmodified cells in the synthesis of nucleosides [18] and may be used up to five–ten times in reactions performed at 60–65° [30].

The efficient chemical method for the synthesis of *cladribine* consists in the stereospecific glycosylation of sodium salt of 2,6-dichloropurine with 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranosyl chloride leading to the formation of the corresponding N^9-β-D-nucleoside as the main product (58%), along with the N^9-α-D-isomer (13%) [31][32]. Treatment of the former with NH_3 afforded *cladribine* (71%) [31][32]. Two enzymatic approaches have been used for the preparation of *cladribine*. First of them consists in the direct transfer of the 2-deoxyribofuranose residue from thymidine to 2Cl-Ade catalyzed by partially purified *trans*-*N*-deoxyribosylase [33]. The other, which seems to be advantageous over the former, makes use of PNPase and UPase of *E. coli* cells (*Table 1*).

In the present study, we have essentially optimized the conditions of the reaction and employed the more efficient biocatalyst GA-*E. coli* BMT 4D/1A (*Table 1*). The enzymatic transdeoxyribosylation studied here includes two coupled reversible reactions, which occur *in situ*: *i*) the phosphorolysis of the glycosyl donor, dGuo, in the presence of inorganic phosphate, resulting in the formation of guanine (Gua) and the key intermediate 2-deoxy-α-D-ribofuranose-1-*O*-phosphate (**3**), and *ii*) the condensation of the latter with 2Cl-Ade with the release of phosphate affording the desired *cladribine* (**4**; *Scheme 1*). Both reactions are catalyzed by PNPase. From the experimental viewpoint, the reaction was conducted in K-phosphate buffer (10 mM; pH

Scheme 1



1 (dGuo) **2** (2Cl-Ade) **3** **4** (*Cladribine*)

Biocatalyst: glutaraldehyde-treated *E. coli* BMT 4D/1A cells.

Table 1. *Enzymatic Synthesis of* Cladribine (**4**)

Donor (donor/acceptor mM concentrations)	Catalyst ([mg/ml])	Time/Temp. ([h/°])	Yield of the reaction [%] (isolated yield [%] of **4**)	Bioreactor productivity [g/l/h]	Ref.
2′-Deoxyadenosine[a]) (30 : 5)	*E. coli* ATCC 5275 (*ca.* 10)	5 (37)	56 ([b]))	0.16	[34]
2′-Deoxyuridine[c]) (20 : 15)	*E. coli* SPT$^-$ (*ca.* 12)	16 (37)	(67)	0.18	[29]
2′-Deoxyguanosine (15 : 5)	GA-*E. coli* BMT 1D/1A (*ca.* 0.2)	21 (60)	65 (54)	0.04	[35]
2′-Deoxyguanosine (120 : 40)	GA-*E. coli* BMT 4D/1A (*ca.* 0.8)	7 (65)	95 (81)	1.55	This study

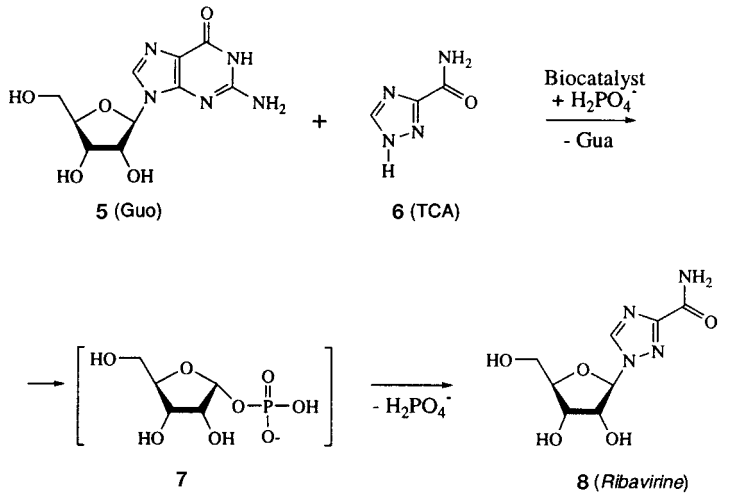
[a]) The 2-chloroadenosine was used as a source of 2Cl-Ade after *in situ* phosphorolysis by PNPase, followed by the enzymatic reaction of 2Cl-Ade with 2-deoxy-α-D-ribofuranose-1-*O*-phosphate resulting from the dAdo phosphorolysis, eventually affording *cladribine*; an excess of dAdo was transformed into 2′-deoxyinosine by the treatment with adenosine deaminase (ADase) for 30 min at 30°. [b]) The isolated yield of individual *cladribine* after crystallization was not indicated. [c]) 2Cl-Ade was not substrate of PNPase of *E. coli* SPT$^-$, and 2-chloro-N^6-(dimethylaminomethylene)adenine was, therefore, used as an acceptor of the 2-deoxypentofuranose residue; alginate gel-entrapped cells were used as a biocatalyst.

7.5) at 65° and separated into two steps. First, the reaction was continued for 4 h, affording the main quantity of **4** (75–80%), then the reaction mixture was stored at +4° for 16–18 h, the biocatalyst as well as dGuo and 2Cl-Ade were filtered off, whereas the most of **4** remained in the filtrate. The second step was conducted under the same conditions in a fresh buffer with the biocatalyst, dGuo, and 2Cl-Ade filtered off previously, giving rise to the formation of an additional quantity of **4** (15–20%). Finally, **4** was purified by silica-gel column chromatography to give, after crystallization from EtOH, the desired compound in 81% isolated yield.

Ribavirin was first synthesised in the 1972 by the chemical glycosylation method [36]. The synthesis is multistep and leads to the formation of two regioisomers that have been separated into individual compounds by chromatography. It should be stressed that only one isomer, *ribavirin*, displayed a broad spectrum of antiviral activity, whereas the other regioisomer is devoid of any biological activity. As distinct from the chemical synthesis, biotechnological process consists in the enzymatic regioselective transfer of the ribofuranose moiety of readily available and inexpensive natural ribonucleosides to the heterocyclic base (**6**, TCA), affording the desired *ribavirin* (**8**; *Scheme 2*). Data on the biotechnological methods are collected in *Table 2*.

As in the case of the *cladribine* (**4**) synthesis, the enzymatic transribosylation of TCA consisted in two coupled reversible reactions: *i*) the phosphorolysis of the glycosyl donor, Guo, in the presence of inorganic phosphate, resulting in the formation of Gua and the key intermediate α-D-ribofuranose-1-*O*-phosphate (**7**), and *ii*) the condensation of the latter with TCA with the release of phosphate, affording the desired *ribavirine* (**8**; *Scheme 2*). Again, both reactions were catalyzed by PNPase. However,

Scheme 2



5 (Guo) 6 (TCA) 7 8 (*Ribavirine*)

Biocatalyst: glutaraldehyde-treated *E. coli* BMT 4D/1A cells.

Table 2. *Enzymatic Synthesis of* Ribavirin (**8**) *by the Transglycosylation of TCA* (**6**)

Donor (donor/acceptor mM concentrations)	Catalyst ([mg/ml])	Time/Temp. [h/°]	Yield of the reaction [%] (isolated yield [%] of **8**)	Bioreactor productivity [g/l/h]	Ref.
Uridine (200 : 200)	*Enterobacter aerogenes* AJ-1125 (*ca.* 20)	96 (60)	55 ([a]))	0.28	[37]
Orotidine (300 : 300)	*Erwinia carotovora* AJ-2992 (*ca.* 20)	48 (60)	61 ([a]))	0.93	[38]
Guanosine (300 : 300)	*Brevibacterium acetilicum* ATCC-954 (*ca.* 10)	96 (60)	73 ([a]))	0.56	[39]
N^7-Methylguanosine (1.0 : 0.25)	PNPase (100 units per 1.0 mmol of N^7-Guo)	48 (25)	44 ([a]))	0.06	[40]
Guanosine (300 : 300)	GA-*E. coli* BMT 4D/1A (*ca.* 15)	30 (60)	90 – 92 (67 – 70)	2.26	This study

[a]) The isolation of individual *ribavirin* was not described.

an application of GA-*E. coli* BMT 4D/1A as a biocatalyst led to displacement of the total equilibrium to the formation of **8** (90 – 92%). Purification of **8** was performed by double ion-exchange chromatography (first *Dowex 50* × *8*, H^+ form, and then *Dowex 1* × *8*, OH^- form) to give, after crystallization from EtOH, the desired compound in 67 – 70% isolated yield. Structure of the synthesized compound **8** was established by comparison of the m.p., UV, and ^{1}H-NMR data with those published in [36].

Conclusions. – We have developed a very efficient biocatalyst for the preparation of modified nucleosides. The biocatalyst represents the glutaraldehyde-cross-linked cells

of *E. coli* BMT 4D/1A strain containing very active PNPase, UPase, and TPase. The synthetic potential of the biocatalyst is manifested by the practical preparation of two medicinal drugs *cladribine* and *ribavirin*. Taking into account that the biocatalyst was earlier successfully used for the preparation of 2′-deoxy-2′-fluoroguanosine [25] and 2,6-diamino-9-(3-deoxy-β-D-*erythro*-pentofuranosyl)purine [26], it may be considered as the universal efficient tool for the preparation of modified nucleosides, employing sugar-modified pyrimidine nucleosides as donors of the pentofuranose residue.

Experimental Part

1. *General*. High-performance liquid chromatography (HPLC) was carried out with the *Waters* apparatus (*Waters*, USA) (column *Nova-Pac C-18* (3.9 × 300 mm, *Waters*); *Waters Pump Control Module*, *Waters 515* HPLC pump; *Waters 996* photodiode array detector operating at 208 and 254 nm for analysis of *ribavirin* and *cladribine*, resp.) and an isocratic elution with 3% MeCN (*ribavirin*) and 7% MeCN (*cladribine*) in H_2O (*v*/*v*) at a flow rate 0.7 ml/min (time of analysis was 15 and 30 min, resp.). Guanosine and 2′-deoxyguanosine were purchased from *Fluka* (Switzerland). Column chromatography (CC): silica gel *60 H* (70–230 mesh ASTM; *Merck*, Germany). TLC: *Silufol* UV_{254} aluminum sheets silica gel *60* F_{254} (*Merck*, Germany). Solvent systems for TLC: $CHCl_3$/EtOH 4:1 (*v*/*v*) (*A*), BuOH/25% aq. NH_2 7:2 (*v*/*v*) (*B*). M.p.: *Boetius* apparatus (Germany); not corrected. UV Spectra were measured with *Specord M-400* spectrometer (*Carl Zeiss*, Germany). ^{1}H- and ^{13}C-NMR spectra were measured at 200.13 and 50.325 MHz, resp., at 23° on an *AC-200* spectrometer equipped with an *Aspect 3000* data system (*Bruker*, Germany); δ values are in ppm downfield from internal Me_4Si (^{1}H, ^{13}C); coupling constants *J* are given in Hz; assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments.

2. *Microorganisms*. The whole *E. coli* BMT-4D/1A cells were used as the source of PNPase. The *E. coli* BMT-4D/1A cells have been selected according to the technique offered by *Munch-Petersen et al.* [41]. They carry two regulatory mutations (cytR and deoR) and are able to synthesize constitutively nucleoside-catabolizing enzymes. The biomass of *E. coli* BMT-4D/1A cells possessing high PNPase activities was prepared as described in [18].

3. *Glutaraldehyde Treatment*. Cell suspension (4% dry weight) in 0.1M phosphate buffer (pH 7.0) was cooled to 4°. A 25% aq. soln. of GA was added with stirring to give a final concentration of 0.75%. Afterwards, the suspension was incubated for 15 min at the same temp. Then the cross-linked cells were washed free of GA by centrifugation five times with 30 volumes of 10 mM phosphate buffer (pH 7.0).

4. *Synthesis of 2-Chloro-2′-deoxyadenosine* (=*Cladribine*; **4**). To the heterogeneous mixture (250 ml; 10 mM K-phosphate buffer, pH 7.5) consisting of dGuo (**1**; 8.0 g, 0.03 mol) and 2Cl-Ade (**2**) [42] (1.7 g, 0.01 mol) at 65°, the biocatalyst (final concentration 0.08% that corresponds to *ca*. 200 mg of the cells calculating for dry weight) was added, and the reaction mixture was incubated at 65° for 4 h with gentle stirring. The formation of the product was monitored by TLC on *Silufol* UV_{254} plates (*A*; double elution): the R_f values for dGuo, 2Cl-Ade, and **4** are 0.13, 0.33, and 0.23, resp. The products were eluted from the TLC plates with H_2O and quantified spectrophotometrically; under indicated conditions, the formation of **4** attained 75–80%. The reaction mixture was stored at +4° for 16–18 h, the formed precipitate, which contained the biocatalyst, as well as dGuo and 2Cl-Ade according to TLC, was filtered off and washed with cold (+4°) H_2O (2 × 40 ml). The filtrate and washings were combined (first H_2O phase), the residue was suspended in K-phosphate buffer (50 ml; 10.0 mM, pH 7.5), and the mixture was incubated again at 65° for 3 h with gentle stirring. The formation of an additional quantity of **4** (15–20%; determined spectrophotometrically) was monitored.

The biocatalyst was removed from the reaction mixture by centrifugation (8000 × g, 5 min). The precipitate was suspended in H_2O (40 ml) with stirring for 15 min, and the biocatalyst was removed as described above. Two supernatants were combined with the first H_2O phase, evaporated to dryness, and the crude **4** was purified by silica-gel CC as described in [23]. The fractions containing individual **4** were combined and evaporated to dryness, the residue was crystallized from EtOH to afford **4** (2.3 g, 81% based on 2Cl-Ade taken into reaction) as a white powder that was identical with the earlier synthesized compound [23] in all respects (TLC, UV, and ^{1}H-NMR). HPLC: t_R 15.39 min (purity 99.60%).

5. *1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide* (**8**). The reaction mixture (100 ml; 10 mM K-phosphate buffer, pH 7.0) consisting of the biocatalyst (final concentration 1.5% that corresponds *ca*. 15 mg/ml of the cells calculating for dry weight), Guo (**5**; 8.5 g, 0.03 mol), and TCA (**6**) [43] (3.36 g, 0.03 mol)

was incubated at 60° for 30 h with gentle stirring. The formation of the product was monitored by TLC on *Silufol* UV_{254} plates (*B*): the R_f values for TCA, **8**, and Guo are 0.22, 0.15, and 0.11, resp. The products were quantified spectrophotometrically, and, under indicated conditions, the formation of the desired compound **8** attained 90–92%. The cells were removed from the reaction mixture by centrifugation (8000 × g, 10 min), washed with H_2O (2 × 40 ml) with the subsequent withdrawal of the cells by centrifugation, and the combined supernatants were loaded onto a column (4.0 × 20.0 cm; *Dowex 50* × *8*, 200–400 mesh; H^+ form). The column was washed with H_2O, slightly acidic (pH 3.0–4.0) *ribavirin*-containing fractions (TLC control) were combined (*ca.* 300 ml), and loaded onto a column (2.5 × 20.0 cm; *Dowex 1* × *8*, 200–400 mesh; OH^- form). The column was washed with H_2O, the *ribavirin*-containing fractions (TLC control) were combined (*ca.* 1.0 l), and evaporated to dryness. The residue was dissolved under reflux in EtOH (70–80 ml), filtered through a glass filter, allowed to cool to r.t. and left in a refrigerator overnight. Crystalline product was filtered off, washed with cold EtOH, and dried at 60° under vacuum to give **8** (4.91–5.13 g, 67–70%; TLC- and HPLC pure). M.p. 176–178°. UV (H_2O): λ_{max} 207 (11600) ([36]: m.p. 166–168° (from aq. EtOH); 174–176° (from EtOH)). HPLC: t_R 3.99 min (purity 99.46%). ^{1}H-NMR ((D_6)DMSO): 8.90 (*s*, H–C(5)), 7.90 (br. *s*, 1 H, 1 H of the $CONH_2$ group presumably H-bonded with N(2)); 7.64 (br. *s*, 1 H, non-H-bonded H of the $CONH_2$ group); 5.82 (*d*, *J*(1′,2′) = 3.9, H–C(1′)); 5.62 (*d*, *J*(H–C(2′, OH–C(2′)) = 5.5, OH–C(2′)); 5.24 (br. *s*, *J*(H–C(3′), OH–C(3′)) = 5.5, OH–C(3′)); 4.98 (*t*, *J*(H–C(5′), OH–C(5′)) = 5.5, OH–C(5′)); 4.38 (*m*, *J*(2′,3″) = 4.6, H–C(2′)); 4.18 (*m*, *J*(3′,4″) = 4.8, H–C(3′)); 3.98 (*m*, *J*(4′,5′) = *J*(4′,5″) = 5.0, H–C(4′)); 3.60 (*m*, *J*(5′,5″) = 12, H–C(5′)); 3.48 (*m*, H–C(5″)).

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