Application of the phosphoramidate ProTide approach to the antiviral drug ribavirin

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ABSTRACT

Ribavirin is a nucleoside analogue with broad antiviral activity. Here we report the synthesis and biological evaluation of novel ribavirin ProTides designed to deliver the bioactive ribavirin monophosphate into cells. Some of the compounds display activity similar to the parent nucleoside against a range of viruses. Enzymatic, cell lysate and preliminary modeling studies have been performed to investigate the lack of enhancement of potency by the ProTides, and these indicate a failure at the final, amino acid cleavage step in the ProTide activation process, leading to inefficient release of the nucleoside monophosphate.

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1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (1, Fig. 1) is a nucleoside analogue with antiviral activity against a number of DNA and RNA viruses in vitro and in vivo.1 Ribavirin has been approved for the treatment of respiratory syncytial virus2 and forms part of the only approved combination therapy for hepatitis C virus (HCV) infection.3 The agent is converted into its 5'-monophosphate form (RMP) by intracellular phosphorylation mediated by adenosine kinase, followed by further phosphorylation to the di- and triphosphate (RDP and RTP).4 Its antiviral mode of action is not completely understood and, depending on the virus examined, several mechanisms have been proposed: (i) RMP inhibits the cellular enzyme inosine monophosphate dehydrogenase (IMPDH), leading to reduced GTP pools,5,6 which may play a synergistic role in promoting the incorporation of ribavirin into viral RNA;7 (ii) RTP inhibits viral RNA synthesis by inhibition of the viral polymerase,8 (iii) 5'-capping of viral mRNAs is blocked by inhibition of the viral 2'-O-methyltransferase (by RTP) or viral guanylyl transferase (by RMP),9 (iv) incorporation of ribavirin into this 5'-cap impairs their function and (v) when incorporated into viral RNA, ribavirin induces an accumulation of mutations, which, if lethal, impede viral replication (so called ‘error catastrophe’).10

Finally, ribavirin has been attributed diverse immunomodulatory activities.11,12 Whatever the precise mechanism, it is clear that phosphorylation of ribavirin to the mono- and perhaps triphosphate forms are crucial for its antiviral activity. In at least some human cell types, formation of the monophosphate by adenosine kinase was shown to be the rate-limiting step in the activation pathway.13

In order to bypass the phosphorylation of antiviral nucleoside analogues, several strategies have been introduced, including the arylxy phosphoramidate ProTide technology, which our laboratories have introduced. The aim of this concept is to synthesize lipophilic prodrugs of the nucleoside monophosphate, which, after passive diffusion and enzymatic cleavage, release the free monophosphate at high intracellular concentrations. The structural motif of the prodrug consists of an aryl moiety and an amino acid ester to mask the negative charges of the nucleoside monophosphate. The ProTide approach has been successfully applied to several antiviral nucleosides such as didehydrodideoxythymidine (d4T),14,15 (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU),16 abacavir,17 4'-azidouridine,18 and acyclovir.19,20

Figure 1. Structure of ribavirin.
2. Results

2.1. Chemistry

The designed compounds carry 1-naphthyl as the arylxoy group, benzyl as the ester function, and varying amino acid moieties including glycine, alanine and phenylalanine.

Ribavirin (1) was obtained (Scheme 1) by deprotection using methanolic ammonia of the fully protected nucleoside 4, which was synthesised by condensation of 1,2,3,5-tetra-acetyl-D-ribofuranose (2) and 1,2,4-triazole-3-carboxylate (3) in the presence of bis(p-nitrophenyl)phosphate. In order to improve the poor solubility of ribavirin and to prevent formation of side-products in the following step, the 2' and 3' positions of the sugar were protected with a cyclopentylidene group giving the first synthon 5.

The synthesis of the second synthon, the naphthyloxy-phosphorochloridate, (Scheme 2) involves phosphorylation of 1-naphthol with phosphorus oxychloride obtaining the corresponding phosphorodichloridate (6), which was then coupled with the appropriate amino acid benzyl ester salts to give the desired compounds (7–9).

The final coupling (Scheme 3) was performed using tert-butyl magnesium chloride (1.0 M solution in tetrahydrofuran) following the Uchiyama procedure to give compounds 10–12 in yields between 15% and 44%. The final products 13–15 were obtained by deprotection of the cyclopentylidene group with 60% formic acid overnight. Yields of the final compounds were between 17% and 63%.

2.2. Antiviral activity

The synthesised compounds were evaluated for their ability to inhibit the replication of several viruses. All compounds displayed increased log P values compared to ribavirin, as evidenced by C log P values (Table 1). This may suggest enhanced cellular permeability, at least by passive diffusion. Although active, the ProTides did not show any marked improvement of activity (Table 1) compared to the parent compound, their antiviral concentrations being, at best, in the 14–100 μM range. In the case of respiratory syncytial virus, for which ribavirin is clinically approved, the alanine (13) and glycine (15) ProTides were about threefold less active than ribavirin (1). Compound 13 proved equipotent to ribavirin in inhibiting HCV subgenomic replicon replication and both ProTides proved equipotent to ribavirin for vesicular stomatitis virus. ProTide 13, but not 15, was active against influenza virus (its potency being ~2-fold lower than that of ribavirin). Compound 15 proved 3–5-fold superior to ribavirin in inhibiting vaccinia virus and Punto Toro virus replication. For all the viruses tested, the phenylalaninyl ProTide 14 had no or borderline activity at the highest concentration tested (100 μM).

All three ProTides were non-toxic to uninfected cells at 100 μM, as was ribavirin. No activity (antiviral EC$_{50}$ >100 μM) was observed against parainfluenza-3-virus, reovirus-1, sindbis virus, Coxsackie virus B4, HIV-1, HIV-2, herpes simplex virus (type 1, wild-type or thymidine kinase-deficient, or type 2) (HSV), cytomegalovirus, feline corona virus and feline herpes virus (data not shown).

In order to understand the relatively poor activity of these ProTides, which is suggestive of ineffective activation, we performed enzymatic and preliminary molecular modeling studies.

2.3. Enzymatic study

The proposed activation mechanism of phosphoramidate produgs (Fig. 2) involves initial hydrolysis of the carboxylic ester by an esterase or carboxypeptidase type enzyme, and subsequent spontaneous cyclisation with displacement of the arylxoy group, followed by opening of the unstable anhydride ring mediated by water. The last step in the bioactivation is considered to involve the hydrolysis of the P–N bond by a phosphoramidase-type enzyme.

An enzymatic study using carboxypeptidase Y was performed in order to investigate whether ribavirin ProTides can be metabolized under these conditions. Compound 13 was incubated with carboxypeptidase Y in acetone–d$_6$ and Trizma buffer (pH 7.6) and the enzyme reaction was followed by $^{31}$P NMR. The spectra (Fig. 3) shows fast metabolism of the starting material ($\Delta \rho = 4$) to the putative intermediate (18) ($\Delta \rho = 7$), the reaction being completed within 1.5–2 h. The suggestion that the reaction product is structure (18) is based on two factors. Firstly, while (13) contains a chiral phosphorus and displays two phosphorus signals in the NMR, the ($\Delta \rho = 7$) product elicits only one signal, suggestive of an achiral phosphorus, as in (18). Secondly, other reports of amino aryl phosphates such as (18) indicate a chemical shift of this magnitude. Close examination of the early (3–30 min) spectra shows a double peak at $\Delta \rho <5$, indicative of a chiral phosphorus and con-
sient with the proposed activation pathway. This species is considered to be the initial cleavage product (16) on the basis of similar analogues.26 This enzyme experiment indicates that the first activation step of ProTide 13 is sufficiently efficient.

### 2.4. Molecular modeling studies

Preliminary molecular modeling studies using docking techniques were performed in order to estimate whether ribavirinL-alanine phosphate (18) could be a substrate for the human HINT (I) enzyme, which is considered to catalyze the P–N cleavage reaction.27 These docking studies, performed by a published method,28 revealed that ribavirinL-alanine phosphate is not able to bind to the catalytic site of the HINT (I) enzyme in a productive orientation (Fig. 4). Namely, the simulation results show that the triazole base is placed in the proximity of the catalytic core, in a position that should be occupied by the phosphoramidate group. This may provide a possible explanation for the poor activity of the ribavirin ProTides.

### 2.5. Metabolism in cell lysates

To further support the modeling study, the prodrugs of ribavirin were chosen for examination of their stability/conversion in concentrated CEM, HeLa and MDCK cell extracts (Fig. 5). In case of 13 and 15, the two diastereoisomeric prodrug forms could be distinguished whereas we were unable to separate the isomers of 14 by HPLC.

Interestingly for 13, a different preference of conversion of the two stereoisomers was observed. One of the isomers (tR: 19.3 min) had almost to be completely converted first to the new metabolite (tR: 20.2 min) (conversion virtually complete (C21 95%)) before the second isomer (tR: 18.8 min) started to be significantly converted to the same metabolite. This trend has also been observed for 15 although the conversion within the time-frame of the experiment was much slower (10% conversion of isomer tR 18.8 min to tR 20.2 after 60 min of the incubation whereas isomer tR 18.5 levels were virtually unaltered).

For prodrug 14 both diastereoisomers (not separable) were most efficiently converted (80% within 20 min) to metabolite (tR: 20.2 min), followed by 13, whereas 15 was the least efficiently converted prodrug in the presence of the CEM cell extract. Also, in all cases, the metabolite that accumulated, proved similar for the three different prodrugs (tR: 20.2 min). Only the isomers of 15 converted slowly to two other (most likely corresponding isomeric) metabolites with earlier retention times (tR: 15.3 and 15.6 min) (only observed in MDCK cell extracts) than the parent compound (tR: 18.5 and 18.8 min). In HeLa cell extracts, a less polar metabolite (tR: 19.8 min) of 15 started to be formed (traces of this metabolite have also been observed in the CEM and MDCK incubation experiments).
The fact that the most prominent metabolite of the three prodrugs were of similar nature ($t_R: 20.2$ min) is somewhat unexpected since it suggests that the (variable) amino acyl part of the molecule had to be removed while keeping a lipophilicity that is very comparable with the parent prodrugs. The nature of this metabolite is currently unclear. It was also notable that, irrespective of the nature of the prodrug and the origin of the cell extract, in no case were significant levels of ribavirin-MP or polar ribavirin-MP metabolites, or ribavirin itself detected. It is currently unclear why no more polar metabolites are observed upon cell extract incubation, as noted above in the cathepsin assay and as is the case with other purine or pyrimidine nucleotide phosphoramidate ProTides studied in the past under identical experimental conditions. More investigations are required to clarify this issue. However, the lack of liberation of ribavirin monophosphate correlates with the somewhat poor antiviral activity noted here, and supports the modeling predictions on HINT. It may be that the unusual base present in ribavirin precludes the usually efficient monophosphate delivery by the ProTide motif.

3. Conclusion

A series of novel ribavirin ProTides has been synthesized. No improvement or expansion of the antiviral activity of the parent nucleoside has been obtained. A likely explanation for this lack of activity is the poor activation of the ProTide to the free monophosphate, as evidenced by the cell lysate incubation studies. Our enzyme studies indicated that the first step in the activation of these ribavirin ProTides is efficient, but that subsequent amino acid cleavage to liberate the necessary free 5′-monophosphate appear impeded in this case. Both our preliminary molecular modeling data with the HINT enzyme, and our cell lysate incubation studies support this conclusion.
4. Experimental section

4.1. Biology

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK\(^{-}\)) HSV-1 KOS strain resistant to ACV (ACV\(^{r}\)), herpes simplex virus type 2 (HSV-2) strain G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Reoivirus-1, Sindbis, Punta Toro, influenza virus type A (H1N1, H3N2) and type B and feline corona virus. The antiviral, other than anti-HIV, assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cervix carcinoma cells (HeLa), Crandel fe-

line kidney cells (CFKC) or Madin-Darby canine kidney cells (MDCK). Briefly, confluent cell cultures in microtitre 96-well plates were inoculated with 100 CCID50 of virus (1 CCID\(_{50}\) being the virus dose to infect 50% of the cell cultures). After a 1–2 h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC\(_{50}\) or compound concentration required to reduce virus-induced cytopathicity by 50%. Cytotoxicity of the test compounds was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that caused a microscopically detectable alteration of cell morphology.

To examine inhibition of HIV-induced cytopathicity in CEM cells, human CEM cell cultures (~3 \times 10^5 cells mL\(^{-1}\)) were infected with ~100 CCID50 HIV-1([Hb] or HIV-2(ROD) per mL and seeded in 96-well (200 \mu L/well) microtitre plates, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, syncytia formation was examined microscopically in the CEM cell cultures. EC\(_{50}\) values were determined as described above.

Huh 7 cells carrying subgenomic HCV replicon \(_{I389luc-ubi-neo/NS3-3/5.1\text{[Huh 5–2]}\}) were employed to evaluate the anti-HCV activity and were kindly provided by R. Bartenschlager, (University of Heidelberg, Heidelberg, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Merelbeke, Belgium) supplemented with 10% heat-inactivated foetal calf serum (Integro, Zaandam, The Netherlands), 1x MEM Non Essential Amino Acids Solution without L-Glutamine (Gibco), 100 IU of penicillin/mL and 100 \mu g of streptomycin/mL (Gibco) and 250 \mu g/mL G418 (Geneticin\(^{\text{\[Selective Antibiotic, Gibco.}}\)). Huh 5–2 cells were seeded at a density of 5 \times 10^3 cells per well in 96-well cell culture plates in tissue culture treated white 96-well view plates [(Packard, Canberra, Canada)] in complete DMEM without G418. Antiviral assays were carried out as reported earlier. Read-out for Huh

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**Figure 4.** Docking of compound 18 within the catalytic site of human HINT (I) enzyme.

**Figure 5.** Cell lysate metabolism of 13–15. For details see experimental section. Conversion of prodrugs 13–15 (upper–lower) in the presence of human T-lymphocyte CEM, human cervix carcinoma HeLa and canine Madin-Darby kidney cell extracts (left to right). The levels of the parent prodrug molecules and their conversion products are given as percentage of total amounts of parent compound and metabolites. The different compounds in the incubation mixtures are shown as their Rt values (retention times) in the HPLC chromatograms.
5–2 cells was a luciferase assay. EC50 was defined as the concentration of compound that reduced the firefly luciferase signal by 50%.

The cytostatic compound concentration was calculated as the CC50, or the compound concentration required to reduce cell proliferation by 50% relative to the number of cells in the untreated controls. CC50 values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds.

4.2. Chemistry

4.2.1. General

Anhydrous solvents were bought from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on Silica Gel 60-F254 precoated aluminium plates and visualised under UV (254 nm) and/or with 31P NMR spectra. Column chromatography was performed on silica gel (35–70 μm). Proton (1H), carbon (13C) and phosphorus (31P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were auto-calibrated to the deuterated solvent peak and all 13C NMR and 31P NMR were proton-decoupled. High resolution mass spectra was performed as a service by Birmingham University, using electrospray (ES). CHN microanalysis were performed as a service by the School of Pharmacy at the University of London.

4.2.2. Standard procedure 1: synthesis of phosphorochloridates 7–9

To a stirred solution of 1-naphthyl dichlorophosphate 6 (1.00 mol/eq) and the appropriate amino acid ester salt (1.00 mol/eq) in anhydrous DCM was added, dropwise at −78 °C under an argon atmosphere, anhydrous TEA (2.00 mol/eq) and the reaction mixture was stirred at room temperature for 2 h. Following the addition the reaction mixture was stirred at −78 °C for 1 h, then at room temperature for 2 h. Formation of the desired compound was monitored by 31P NMR. After this period the solvent was removed under reduced pressure and the residue triturated with dry diethyl ether. The precipitate was filtered under nitrogen and the solution was concentrated to give an oil. The aryl phosphorochloridates synthesised were purified by flash column chromatography (eluting with ethyl acetate/petroleum ether in different proportions).

4.2.3. Standard procedure 2: synthesis of phosphoramidates 10–12

To a stirring suspension/solution of 5 (1.00 mol/eq) in anhydrous THF was added dropwise under an argon atmosphere 1BuMgCl (2.00 mol/eq) and the reaction mixture was stirred at room temperature for 30 min. Then was added dropwise a solution of the appropriate phosphorochloridate (1.10 mol/eq) in anhydrous THF. The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions.

4.2.4. Synthesis of 1-naphthyl dichlorophosphate (6)

To a stirred solution of 1-naphthol (4.00 g, 27.74 mmol) in dry diethyl ether (60 mL), under an argon atmosphere, were added PhCl (5.50 mL, 27.74 mmol) and anhydrous TEA (3.87 mL, 27.74 mmol) was then added dropwise, at −78 °C. Following the addition, after 30 min at −78 °C, the reaction mixture was stirred at room temperature overnight. After 31P NMR, the solvent was removed under reduced pressure and the residue was triturated with dry diethyl ether. The precipitate was filtered, and the organic phase was removed under reduced pressure to give a yellow oil (95%, 6.91 g). 31P NMR (CDCl3, 202 MHz): δ 3.72. 1H NMR (CDCl3, 500 MHz): δ 8.02–8.00 (1H, m, H-8), 7.81–7.80 (1H, m, H-5), 7.72–7.70 (1H, m, H-4), 7.54–7.45 (4H, m, H-2, H-3, H-6, H-7).

4.2.5. Synthesis of 1-naphthyl[benzoyl-L-alaninyl]-phosphorochloridate (7)

Prepared according to Standard Procedure 1, 6 (6.91 g, 26.48 mmol), L-alanin benzyl ester HCl (9.30 g, 26.48 mmol), anhydrous TEA (7.40 mL, 52.96 mmol) in anhydrous DCM (100 mL). The reaction mixture was stirred at −78 °C for 1 h, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 5/5 to give a yellow oil (72%, 7.68 g). 31P NMR (CDCl3, 202 MHz): δ 8.14, 7.88. 1H NMR (CDCl3, 500 MHz): δ 7.99–7.25 (12H, m, Naph, OCH2Ph), 5.15–5.07 (2H, m, CH2Ph), 4.30–4.23 (1H, m, CHCH2), 1.49–1.46 (3H, m, CHCH3).

4.2.6. Synthesis of 1-naphthyl[benzoyl-L-phenylalaninyl]-phosphorochloridate (8)

Prepared according to Standard Procedure 1, 6 (0.78 g, 3.00 mmol), L-phenylalanin benzyl ester HCl (0.87 g, 3.00 mmol), anhydrous TEA (0.84 mL, 6.00 mmol) in anhydrous DCM (20 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give a yellow oil (40%, 0.57 g). 31P NMR (CDCl3, 202 MHz): δ 8.32, 8.19. 1H NMR (CDCl3, 500 MHz): δ 8.04–6.90 (17H, m, Naph, CH2Ph, OCH2Ph), 5.20–5.11 (2H, m, OCH2Ph), 4.64–4.52 (1H, m, CHNH), 3.22–3.08 (2H, m, CH2Ph).

4.2.7. Synthesis of 1-naphthyl[benzoyl-glycyl]-phosphorochloridate (9)

Prepared according to Standard Procedure 1, 6 (0.78 g, 3.00 mmol), glycine benzyl ester HCl (1.01 g, 3.00 mmol), anhydrous TEA (0.84 mL, 6.00 mmol) in anhydrous DCM (20 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give a yellow oil (73%, 0.85 g). 31P NMR (CDCl3, 202 MHz): δ 8.90. 1H NMR (CDCl3, 500 MHz): δ 8.02–7.30 (12H, m, Naph, OCH2Ph), 5.17 (2H, s, OCH2Ph), 4.24–4.19 (1H, m, NHCH2), 4.00–3.96 (2H, m, NHCH2).

4.2.8. Synthesis of 2,3-[0.0-cyclopentylidene-ribavirin-5-[1-naphthyl[benzoyl-L-alaninyl]]] phosphate (10)

Prepared according to Standard Procedure 2, from 5 (0.20 g, 0.64 mmol) in anhydrous THF (6 mL), BuMgCl (1.0 M THF solution, 1.30 mL, 1.30 mmol), 7 (0.29 g, 0.71 mmol) in anhydrous THF (6 mL), and the reaction mixture was stirred at room temperature overnight. After the solution was concentrated and the residue purified by column chromatography eluting with DCM/MeOH = 98/2, to give a white solid (32%, 0.14 g). 31P NMR (CDCl3, 202 MHz): δ 3.44, 3.17. 1H NMR (CDCl3, 500 MHz): δ 8.20, 8.19 (1H, 2s, H-5), 7.97–7.18 (12H, m, Naph + OCH2Ph), 5.94 and 5.85 (1H, 2d, H-1'), 5.03–5.00 (2.5H, m, OCH2Ph, H-2' of one diastereoisomer), 4.78–4.76 (0.5H, m, H-3' of one diastereoisomer), 4.69–4.67 (0.5H, m, H-2' of one diastereoisomer), 4.51–4.49 (0.5H, m, H-3' of one diastereoisomer), 4.47–4.44 (1H, m, H-4'), 4.25–4.20 (1H, m, H-5'), 4.19–4.11 (1H, m, H-5'), 4.07–4.02, 4.00–3.90 (1H, 2m, CHCH2), 1.92–1.87 (2H, m, CH2), 1.67–1.52 (6H, m, 3xCH2), 1.30, 1.27 (3H, 2d, CH2H3).

4.2.9. Synthesis of 2,3-[0.0-cyclopentylidene-ribavirin-5-[1-naphthyl[benzoyl-L-phenylalaninyl]]] phosphate (11)

Prepared according to Standard Procedure 2, from 5 (0.20 g, 0.64 mmol) in anhydrous THF (6 mL), BuMgCl (1.0 M THF solution, 1.30 mL, 1.30 mmol), 8 (0.34 g, 0.71 mmol) in anhydrous THF (7 mL), and the reaction mixture was stirred at room temperature
overnight. After this period the solution was concentrated and the residue purified by column chromatography eluting DCM/MeOH = 98/2, to give a white solid (44%, 0.22 g). \(^{31}\text{P}\) NMR (MeOD, chromatography gradient elution of DCM/MeOH = 96/4 then 95/5, solution was concentrated and the residue purified by column chromatography was stirred at room temperature overnight. After this period the solution was concentrated and the residue purified by column chromatography eluting with DCM/MeOH = 98/2, to give a white solid (15%, 0.066 g). \(^{31}\text{P}\) NMR (CDCl\(_3\), 2 min 2% acetonitrile (Buffer A); 8 min linear gradient to 20% Buffer A and 80% Buffer B; 10 min linear gradient to 40% Buffer A + 60% Buffer B; 15 min linear gradient to 60% Buffer A + 40% Buffer B; 20 min linear gradient to 100% Buffer A. At each time point 100 lL was withdrawn from the incubation medium and added to 200 lL ice-cold methanol 100% (final concentration: 66%). After 10 min incubation at 4 °C, the reaction mixtures were centrifuged at 13,000 rpm for 10 min and 150 lL of the supernatants were analyzed by HPLC. The retention times for 13 were 19.0 and 19.3 min (both isomers), for 14 was 21.9 min (isomers not separable) and for 15 were 18.5 and 18.7 min (both isomers). Separation of the parent compounds and conversion products was performed on a reverse phase (Lichrospher-60 RP-select B) column (Merk, Darmstadt, Germany) using following gradient: 2 min 2% acetonitrile (Buffer A); 8 min linear gradient to 20% Buffer A + 80% Buffer B (50 mM NaH\(_2\)PO\(_4\) + 5 mM heptane sulfonic acid); 2 min linear gradient to 25% Buffer A and 75% Buffer B; 2 min linear gradient to 35% Buffer A + 65% Buffer B; 8 min linear gradient to 50% Buffer A and 50% Buffer B; 10 min isocratic flow; 5 min linear gradient to 2% Buffer A + 98% Buffer B; 5 min equilibration by 2% Buffer A + 98% Buffer B.

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**Supplementary data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bmc.2010.02.015.

**References and notes**