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Original Scientific Article

Influence of the Acyl Moiety on the Hydrolysis of Quinuclidinium Esters Catalyzed by Butyrylcholinesterase†

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Abstract. Eight chiral esters of quinuclidin-3-ol and butyric, acetic, pivalic and benzoic acid were synthesized as well as their racemic and chiral, quaternary *N*-benzyl derivatives. All racemic and chiral quaternary compounds were studied as substrates and/or inhibitors of horse serum butyrylcholinesterase (BChE). The best substrate for the enzyme was (*R*)-*N*-benzyl butyrate. The rates of hydrolysis decreased in order (*R*)-butyrate >> (*R*)-acetate (7-fold slower) > (*R*)-pivalate (8-fold slower) > (*R*)-benzoate (9-fold slower reaction), while (*S*)-*N*-benzyl esters were much poorer substrates (320 (butyrate) - 4360-fold slower (pivalate) than the appropriate (*R*)-enantiomer). For all (*S*)-*N*-benzyl esters excluding (*S*)-*N*-benzyl acetate inhibition constants were determined (K_a = 3.3−60 μmol dm^{-3}). The hydrolysis of racemic mixtures of *N*-benzyl esters proceeded 1.4 (for acetate) − 5.1 (for benzoate) times slower than that of pure (*R*)-enantiomers of the corresponding concentrations due to the inhibition with (*S*)-enantiomers. Change of the acyl moiety of the substrate effected both activity and stereoselectivity of the BChE.(doi: 10.5562/cca1829)

Keywords: esters of quinuclidin-3-ol, enzymic resolution, butyrylcholinesterase, hydrolysis kinetics, inhibition constants

INTRODUCTION

Azabicyclo[2.2.2]octan-3-ol, its derivatives and other quinuclidine compounds display a broad range of biological activities and pharmaceutical industries have large interest to explore them.[1] Among other activities, they were shown to be potential antidotes against organophosphorus poisoning caused by warfare agents.[2–4] Quinuclidin-3-ol is a chiral compound which contains an asymmetric carbon atom at the position 3 of the bicyclic ring. Since the racemates are regarded with suspicion as pharmaceuticals (enantiomers can have different activity or even toxic effects), the issue of quinuclidin-3-ol resolution has been addressed by using a number of chemical[5–7] and biocatalytic[8,9] methods. One of the enzymes tested as a biocatalyst was butyrylcholinesterase from horse serum (BChE, EC 3.1.1.8.). This enzyme has not been used as much as some other esterases for biotransformations in organic chemistry[10] because of its preference toward positively charged substrates which resemble its best substrate: butyryl choline.[11–13] Structural similarity among choline and quinuclidin-3-ol implied that quinuclidine esters might be good substrates of the enzyme. Thus, it was possible to use BChE for the resolution of racemic nonquaternized quinuclidin-3-yl butyrate[8] and for the hydrolysis of (*R*)- and (*S*)-quinuclidin-3-yl benzoates,[14–16] stereoselectivity of hydrolyses being in favour of the (*R*)-enantiomer.

In this paper the synthesis of quaternary, *N*-benzyl derivatives of racemic, (*R*)- and (*S*)-quinuclidin-3-yl butyrate, acetate, pivalate and benzoate are reported, Figure 1. Quaternization with *N*-benzyl group was used because it can be considered as a protecting group which can be successfully removed afterwards.[17]

All synthesized quaternary compounds were studied as substrates and/or inhibitors of horse serum BChE to determine the potential of BChE as a biocatalyst in kinetic resolution of quinuclidine esters.

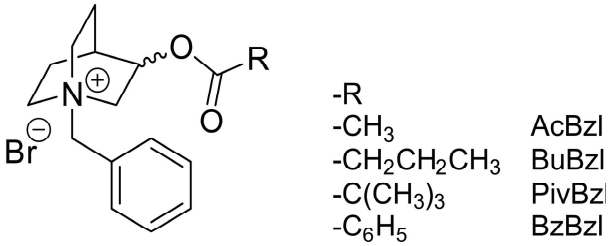

Figure 1. Structures of synthesized quinuclidinium esters.

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EXPERIMENTAL SECTION

Melting points were determined in open capillaries using a Büchi B-540 melting point apparatus and are uncorrected. Specific optical rotation values were determined with an Optical Activity LTD automatic polarimeter AA-10 on 589 nm at ambient temperature (≈24 °C) in methanol. Elemental analyses were performed with a Perkin-Elmer PE 2400 Series II CHNS/O Analyser. IR spectra were recorded with a Perkin-Elmer FTIR 1725 X spectrometer. ^{1}H and ^{13}C 1D and 2D NMR spectra were recorded with Varian XL-GEM 300 spectrometer at room temperature. Chemical shifts are given in ppm downfield from TMS as internal standard. HPLC analyses (Thermo Separation Products, Spectra-SYSTEM 2000) were performed on a RP18 (Waters, SymmetryShield, 5 μm, 3.9×150 mm) column (40 °C). The mobile phase used was water/ methanol/ acetonitrile/ acetic acid/ triethylamine (60/25/15/0.33/0.2), flow rate 1 ml/min. The reactions were carried out in Heidolph UNIMAX 1100 Shaker. BChE (EC 3.1.1.8), type IV-S lyophilized powder from horse serum (Sigma Chemical Co.) was used without further purification. The hydrolysis of esters catalyzed by BChE was monitored by following the production of *N*-benzylquinuclidin-3-ol by HPLC analysis as described previously.[15] All experiments for (*R*)-enantiomers were performed with 1.5×10^{-9} mol dm^{-3} and for (*S*)-enantiomers with 1.5×10^{-8} mol dm^{-3} concentration of BChE. The dissociation constants of enzyme-inhibitor complex for (*S*)-enantiomers were determined from Hunter-Downs plot, using benzoyl choline (BzCh) as a substrate.

Preparation of substrates

(*R*)- and (*S*)- quinuclidin-3-ol were prepared by a resolution procedure as described previously using L- and D-tartaric acid.[5] All esters were prepared according to the procedure described for quinuclidin-3-yl benzoate**.**[14] *N*-quaternary derivatives were prepared by the addition of benzyl bromide (2 equivalents) to the solution of appropriate chiral quinuclidin-3yl ester in dry ether. Synthesis and physical properties of benzoates (BzBzl)[15] and acetates (AcBzl)[18] were described previously.

Quinuclidin-3-yl butyrate, colourless oil, yield 95 %, (*R*)-enantiomer: $[\alpha]_D^{23}$ –22.0° (*c* = 3, EtOH), (*S*)-enantiomer $[\alpha]_D^{23}$ +22.0° (*c* = 3, EtOH); **IR** (NaCl) $\tilde{\nu}$ /cm^{-1}: 2948, 2873, 1731 1457, 1310,1257, 1185, 1081, 979, 779, 753; **^{1}H NMR** ($CDCl_3$) δ / ppm: 0.93 (t, 3H, −C<u>H</u>$_3$, ^{3}J = 7.4 Hz), 1.35−1.95 (m, 6H, H_5, H_8 and −C<u>H</u>$_2$CH$_3$), 1.95−2.01 (m, 1H, H_4), 2.27 (t, 2H, −C<u>H</u>$_2$CH$_2$CH$_3$, ^{3}J = 7,4 Hz), 2.62−2.90 (m, 5H, H_6, H_7 and 1H_2 *cis*), 3.21 (dd, 1H, H_2, ^{2}J = 14.6 and ^{3}J = 8.5 Hz, *trans*), 4.66−4.81 (m, 1H, H_3); **^{13}C NMR** ($CDCl_3$) δ / ppm: 13.41 (−<u>C</u>H$_3$), 18.27 (C_8), 19.20 (C_5), 24.15 (−CH$_2$<u>C</u>H$_2$CH$_3$), 24.91 (C_4), 36.20 (−<u>C</u>H$_2$CH$_2$CH$_3$), 46.16 (C_6), 47.07 (C_7), 55.20 (C_2), 70.62 (C_3), 173.47 (C=O); **ESMS:** *m*/*z* (*m*/*z* calcd. for $C_{11}H_{20}NO_2^+$, 198.14) found 198.2.

3-Butyryloxy-1-benzylquinuclidinium bromide (BuBzl): recrystallization from methanol/ether gave white crystals, yield 94 %; m.p. 169.1−170.0 °C (decomp.); (*R*)-enantiomer **(RBuBzl)**: $[\alpha]_D^{23}$ +19.0° (*c* = 1, EtOH); (*S*)-enantiomer **(SBuBzl):** $[\alpha]_D^{23}$ −19.0° (*c* = 1, EtOH); **UV** (EtOH) λ_{max}/nm: 257, 262 and 269 (log ε / dm^3 mol^{-1} cm^{-1}: 2.50; 2.55 and 2.42); **IR** (KBr) $\tilde{\nu}$ /cm^{-1}: 3022, 2955, 2877, 1726, 1461, 1453, 1378, 1283, 1156, 1002, 980, 706; **^{1}H NMR** ($CDCl_3$) δ / ppm: 0.86 (t, 3H, −<u>C</u>H$_3$, ^{3}J = 7,4 Hz), 1.50−1.65 (m, 2H, −<u>C</u>H$_2$CH$_3$), 1.87−2.49 (m, 5H, H_5, H_8 and H_4), 2,34 (t, 2H, −<u>C</u>H$_2$CH$_2$CH$_3$, ^{3}J = 7,4 Hz), 3.31−4.02 (m, 5H, H_6, H_7 and 1H_2 *cis*), 4.20−4.40 (m, 1H, H_2 *trans*), 5.05−5.21 (m, 3H, −C<u>H</u>$_2$− *bzl* and H_3), 7.25−7.71 (m, 5H, *bzl*); **^{13}C NMR** ($CDCl_3$) δ / ppm: 13.38 (−<u>C</u>H$_3$), 17.99 (C_8), 18.21 (−CH$_2$<u>C</u>H$_2$CH$_3$), 21.20 (C_5), 24.53 (C_4), 35.68 (−<u>C</u>H$_2$CH$_2$CH$_3$), 53.23 (C_6), 53.64 (C_7), 59.74 (C_2), 66.31 (−<u>C</u>H$_2$− *bzl*), 66.69 (C_3), 126.59 (C_1 *bzl*), 129.00 (C_3, C_5 *bzl*), 130.34 (C_4 *bzl*), 133.18 (C_2, C_6 *bzl*), 172.56 (C=O); **ESMS:** *m*/*z* (calcd. for $C_{12}H_{22}NO_2^+$ 288.20) found 288.3.

Anal. Calcd. mass fractions of elements, *w*/%, for $C_{18}H_{26}BrNO_2$ (M_r = 368.31) are C 58.70, H 7.12, N 3.80; found: C 57.21; H 7.03; N 3.89.

Quinuclidin-3-yl pivalate, colourless oil, yield 97 %, (*R*)-enantiomer: $[\alpha]_D^{27}$ +18.4° (*c*=2.0; EtOH); (*S*)-enantiomer $[\alpha]_D^{27}$ −17.9° (*c*=2.0, EtOH); **IR** (NaCl) $\tilde{\nu}$ /cm^{-1}: 2954, 2871, 1725, 1668, 1480, 1457, 1396, 1285, 1163, 1033, 981, 773; **^{1}H NMR** (DMSO-d_6) δ / ppm: 1.19 (s, 9H, (C<u>H</u>$_3$)$_3$C), 1.31−1.77 (m, 4H, H_5 and H_8), 1.85−1.92 (m, 1H, H_4), 2.38−2.76 (m, 5H, H_6, H_7 and H_2 *cis*), 3.09 (dd, 1H, H_2, ^{2}J=14.5 i ^{3}J = 8.2 Hz, *trans*), 4.60−4.67 (m, 1H, H_3); **^{13}C NMR** (DMSO-d_6) δ / ppm: 19.41 (C_5), 24.05 (C_8), 24.98 (C_4), 26.91 ((<u>C</u>H$_3$)$_3$C), 38.28 (<u>C</u>(CH$_3$)$_3$), 45.94 (C_6), 46.97 (C_7), 55.30 (C_2), 70.71 (C_3), 177.46 (C=O); **ESMS:** *m*/*z* (calcd. for $C_{12}H_{22}NO_2^+$ 212.16) found 212.2.

3-Pivaloyloxy-1-benzylquinuclidinium bromide (PivBzl): recrystallization from methanol/ether gave white crystals, yield 86 %; m.p. 166.4−167.4 °; (*R*)-enantiomer (**RPivBzl**): $[\alpha]_D^{28}$ –15.0° (*c* = 1.0, MeOH); (*S*)-enantiomer (**SPivBzl**): $[\alpha]_D^{28}$ +15.2° (*c* = 1.0, MeOH); **UV** (EtOH) λ_{max}/nm: 257, 262 and 269 (log ε/dm^3 mol^{-1} cm^{-1}: 2.50; 2.55 and 2.42); **IR** (KBr) $\tilde{\nu}$ /cm^{-1}: 3032, 2955, 2877, 1729, 1465, 1455, 1376, 1284, 1157, 1005, 990, 704; **^{1}H NMR** (DMSO-d_6) δ / ppm: 1.19 (s, 9H, (C<u>H</u>$_3$)$_3$C-), 1.81−2.08 (m, 4H, H_5, H_8), 2.27−2.28 (m, 1H, H_4), 3.42−3.56 (m, 5H, H_6, H_7, 1H_2 *cis*), 3.87 (dd, 1H, H_2, ^{2}J = 12.9 and ^{3}J = 8.7 Hz, *trans*), 4.61−4.72 (m,

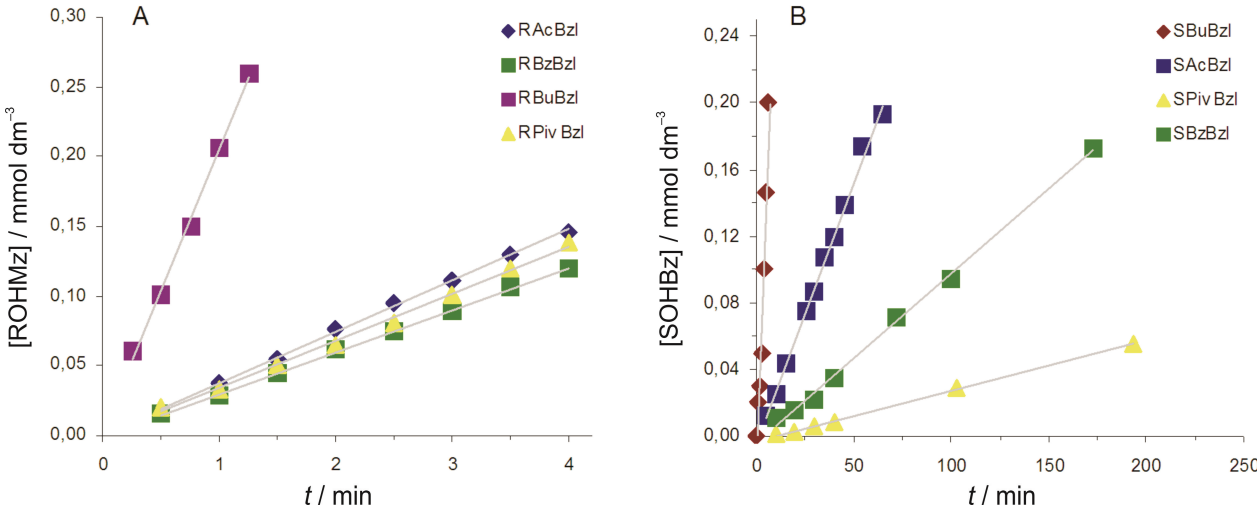


Figure 2. The initial rates of BChE catalyzed hydrolysis of A) (*R*)-esters (A(BChE)/μmol dm^{-3} min^{-1} mg^{-1} equals 2069 for RBuBzl, 280 for RAcBzl, 263 for RPivBzl and 236 for RBzBzl) and B) (*S*)-esters (A(BChE)/μmol dm^{-3} min^{-1} mg^{-1} equals 6.480 for SBuBzl, 0.625 for SAcBzl, 0.063 for SPivBzl and 0.203 for SBzBzl). Monitoring was done by the HPLC analysis of the products. Concentration of all substrates was 4 mmol dm^{-3}. Each data point represents the average value of three measurements.

2H, −C<u>H</u>$_2$− *bzl*), 4.99−5.00 (m, 1H, H$_3$), 7.50−7.59 (m, 5H, *bzl*); **^{13}C NMR** (DMSO-d$_6$) δ/ppm: 18.08 (C$_5$), 20.49 (C$_8$), 24.20 (C$_4$), 26.79 ((<u>C</u>H$_3$)$_3$C−), 38.28 ((CH$_3$)$_3$<u>C</u>−), 52.10 (C$_6$), 53.80 (C$_7$), 59.83 (C$_2$), 65.64 (−<u>C</u>H$_2$− *bzl*), 67.07 (C$_3$), 127.70 (C$_1$ *bzl*), 129.00 (C$_3$, C$_5$ *bzl*), 130.24 (C$_4$ *bzl*), 133.21 (C$_2$, C$_6$ *bzl*), 176.91 (C=O); **ESMS:** *m*/*z* (calcd. for $C_{19}H_{28}NO_2^+$ 302.21) found 302.2.

Anal. Calcd. mass fractions of elements, *w*/% , for $C_{19}H_{28}BrNO_2$ (M_r = 382.34) are C 59.69, H 7.38, N 3.66; found: C 60.38, H 7.12, N 3.59.

RESULTS AND DISCUSSION

Synthesis of quaternary quinuclidinium esters

Chiral (*R*)- and (*S*)-quinuclidin-3-ols were obtained by the resolution of racemic quinuclidin-3-yl acetates with L- and D-tartaric acid.[5] *N*-benzyl derivatives of racemic, (*R*)- and (*S*)-quinuclidin-3-yl acetates, benzoates, pivalates and butyrates were synthesized by the esterification of quinuclidin-3-ol with appropriate anhydride in good yields. Quaternization of racemic and chiral esters with benzyl bromide followed. The structure and purity of all compounds were determined by HPLC, IR, MS, elemental analyses, one- and two-dimensional ^{1}H and ^{13}C NMR.

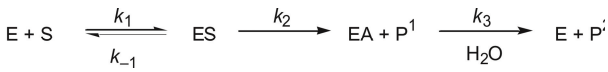
Hydrolyses of quaternary esters with BchE

The overall catalytic process of BChE proceeds in three steps: initial formation of an enzyme–substrate complex, an acylation step, and deacylation by hydrolysis,[19] Scheme 1. In the reaction sequence, E, S, ES, and EA represent free enzyme, substrate, Michaelis complex and acyl-enzyme intermediate, respectively. P^1 (alcohol) and P^2 (acid) are products of the hydrolysis. In the case of tested quinuclidinium esters, 1-benzyl-3-hydroxyquinuclidinium is P1 and P2 are acetic, pivalic butyric and benzoic acid respectively.

All tested chiral esters were substrates of the BChE, Figure 2. Analyses of the initial rates revealed that the hydrolysis of butyric esters was the fastest among all examined enantiomers, and that in general the (*R*)-enantiomers are significantly better substrates for BChE then (*S*)-enantiomers. The rates of hydrolysis for (*R*)-enantiomers decreased in order (*R*)-butyrate >> (*R*)-acetate (7-fold slower) > (*R*)-pivalate (8-fold slower) > (*R*)-benzoate (9-fold slower reaction). The enzyme was even more selective in case of (*S*)-enantiomers: the rates of hydrolysis decreased in order (*S*)-*butyrate* > (*S*)-*acetate* (10-fold slower) > (*S*)-*benzoate* (32-fold slower) > (*S*)-*pivalate* (103-fold slower), Figure 2, chart B. On the other hand, difference in the initial rates for each pair of enantiomers was the biggest for pivalates ($A_{R/S}$=4360), then benzoates ($A_{R/S}$=1180), acetates ($A_{R/S}$=450) and butyrates ($A_{R/S}$=320).

Inhibition of BChE by (S)-enantiomers

Since the rates of hydrolysis of (*S*)-enantiomers were much slower than the rate of hydrolysis of benzoyl choline (BzCh) which can be used to monitor enzyme



Scheme 1.

Table 1. Inhibition constants of BChE by (*S*)-*N*-benzyl esters: the enzyme-inhibitor dissociation constant K_i and standard deviation was obtained from four experiments on average. The dissociation constant of enzyme-inhibitor complex was determined from Hunter-Downs plots. The value for SBzBzl was published previously[15]

Inhibitor	K_m/mmol dm^{-3}	K_i/μmol dm^{-3}
SBuBzl	0,19 ± 0,01	60,5 ± 0,5
SPivBzl	0,79 ± 0,02	12,3 ± 0,2
SBzBzl[15]	1,6 ± 0,6	3,3 ± 0,4

K_m corresponds to $(k_{-1}/k_1 + k_2/k_1)[k_3/(k_2 + k_3)]$

activity, it was possible to determine enzyme-inhibitor dissociation constant K_i. The enzyme-inhibitor dissociation constant K_i was calculated from equation $K_{app}=K_i+(K_a/K_m)\cdot s$, where K_{app} is the apparent enzyme-inhibitor dissociation constant at a given substrate concentration (s) and K_m is the Michaelis constant for the substrate. All (*S*)-esters acted as reversible inhibitors of the enzyme, Table 1. Only SAcBzl inhibited the enzyme when concentrations of BzCh were lower than 0.22 mmol dm^{-3}, and activated the enzyme when the concentration of BzCh was higher, Figure 3. Therefore, it was not possible to determine the enzyme-inhibitor dissociation constant for that compound.

All other (*S*)-esters proved to be μmol dm^{-3} inhibitors, (*S*)-benzoate was the best inhibitor, closely followed by (*S*)-pivalate. (*S*)-butyrate had the lowest affinity toward BChE, Table 1. The calculated values of K_m for the substrate used (BzCh) obtained from kinetic studies with inhibitors can be compared with ones obtained from data without inhibitors (K_m (BzCh) = 0.17±0.01).[14] It can be concluded that SBuBzl competed with BzCh only in the active site while SPivBzl and especially SBzBzl competed with the substrate in the peripheral site as well.

To further explore the hydrolysis reaction of racemic *N*-benzyl quinuclidin-3-ol esters catalysed by BChE, kinetic experiments were carried out to determine the differences in rates of hydrolysis of pure (*R*)-enantiomers (2 mmol dm^{-3} solution) and racemic mixtures which contained the same concentration of (*R*)-enantiomer (4 mmol dm^{-3} solution of the racemate). The obtained values for enzymic activity are presented in Table 2.

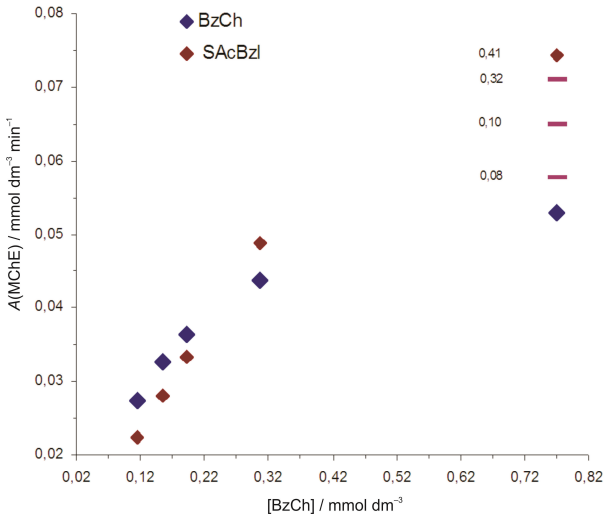


Figure 3. The kinetic study of inhibitory effectiveness of SAcBzl toward BChE in catalyzed hydrolyses of BzCh. Each data point represents the average value of three measurements. Concentrations of SAcBzl are expressed in mmol dm^{-3}.

All reactions of racemate mixtures were slower than the ones of pure (*R*)-enantiomers. The hydrolysis of RBuBzl was 2.0-fold, RPivBzl 2.7-fold faster, while that of RBzBzl was the fastest (5.1-fold) in comparison to the appropriate racemate. These data are in accordance with the measured inhibition constants for (*S*)-enantiomers, SBzBzl beeing the best inhibitor, followed by (*S*)-pivalate and (*S*)-butyrate. It is interesting that in the case of racemic AcBzl there was no effect of activation with present SAcBzl because RAcBzl was the substrate instead of BzCh, Figure 3., in the enzyme activity measurements. The hydrolysis of RAcBzl was 1.4-fold faster when (*S*)-enantiomer was not present, thus the inhibition of SAcBzl was the lowest in magnitude

Table 2. Activity of BChE (A) measured for racemic mixtures and (*R*)-enantiomers of all compounds: 4 mmol dm^{-3} solution of racemate and 2 mmol dm^{-3} solution of (*R*)-enantiomer. Each data point represents the average value of three measurements. $A_{R/racemate}$ corresponds to the ratio of BChE activity for (*R*)-enantiomer and racemate

Substrate	A(BChE)/mmol dm^{-3} min^{-1} mg^{-1}		$A_{R/racemate}$	Inhibition by (*S*)-enantiomer / %
	(R)-enantiomer	*Racemate*		
BuBzl	0.993	0.502	2.0	50
AcBzl	0.184	0.129	1.4	29
PivBzl	0.252	0.094	2.7	63
BzBzl	0.406	0.080	5.1	80

among (*S*)-enantiomers. The BChE catalysed hydrolysis of RBzBzl was slower in case of 4mmol dm^{-3} solution (Fig. 2.) than in case of 2 mmol dm^{-3} solution (Table 2.) indicating inhibition by substrate (RBzBzl) when the higher substrate concentrations are present.

CONCLUSION

We have prepared racemic and enantiomerically pure esters of chiral quaternary *N*-benzyl quinuclidin-3-ol and butyric, acetic, pivalic and benzoic acid. Kinetic studies of BChE-catalyzed ester hydrolysis showed that the reaction proceeds in a stereoselective manner: (*R*)-enantiomers were hydrolyzed preferentially. On the other hand, (*S*)-enantiomers showed much higher affinity toward BChE and all were determined as μmol dm^{-3} inhibitors of the enzyme with the exception of the acetate derivative. It was revealed that there is a great influence of the acyl moiety both on the activity and stereoselectivity of the hydrolysis. The best substrates for the enzyme were butyrates which acyl group is the same as the one in butyrilcholine. Their acyl group fitted the best in the acyl binding site of the enzyme compared to other esters, resulting with the fastest reactions but, at the same time, with the loss of selectivity towards enantiomers. Smaller acetate group and bigger pivalate and benzoate could not realize a maximum possible interactions and the activity of the enzyme was lower. Among (*S*)-enantiomers, the differences in the activity of BChE were even more pronounced and partly related to their inhibitory properties. Effective inhibition of the enzyme indicates that there is a strong non-productive binding of (*S*)-enantiomers which can realize stabilizing contacts within the active site.[14,16]

Due to the satisfactory difference in the rates of hydrolysis of enantiomers, BChE can be used as a biocatalyst in preparations of optically pure quaternary quinuclidin-3-ols. *N*-benzyl group can be efficiently removed by catalytic transfer hydrogenation,[20] thus regenerating chiral quinuclidin-3-ols, precursors for the synthesis of a range of pharmacologically interesting analogues. Consequently, kinetic resolution of racemic *N*-benzyl quinuclidinium esters can be successfully achieved by the stereoselective hydrolysis catalysed with BChE.

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