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Synthesis and biochemical evaluation of highly enantiomerically pure (*R*,*R*)- and (*S*,*S*)-alexidine

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ABSTRACT

Alexidine is in everyday human use as oral disinfectant and contact lens disinfectant. It is used as a mixture of stereoisomers. Since all of alexidine's known biological targets are chiral, the biological activity of any of its chiral stereoisomers could be significantly higher than that of the mixture of stereoisomers. This makes a synthetic methodology for obtaining the individual enantiomers of the chiral diastereoisomer highly desirable. Here, we describe the first synthesis of both enantiomers of alexidine in high enantiomeric purity, and demonstrate their activity against the protein–protein interaction between the anti-apoptotic protein Bcl-x_L and the pro-apoptotic protein Bak.

1. Introduction

Alexidine[1] is a bisbiguanide which is currently in widespread human use as the active ingredient of the oral disinfectant Esemdent®, and the contact lens disinfectant Complete RevitaLens®. The antibacterial activity of alexidine is thought to be based on interactions between the positively charged biguanide moieties and the negatively charged phospholipids on the bacterial membrane.[2] Recently, the mitochondrial dual specificity phosphatase PTPMT1 has been identified as another target of alexidine.[3] Other activities of alexidine reported in the literature include those against tumor cells derived from oral tissues[4] and multiple myeloma.[5]

We recently discovered that alexidine inhibits protein–protein interactions mediated by the anti-apoptotic Bcl-2-family protein Bcl-x_L.[6] Bcl-x_L is overexpressed in a large number of human tumors and has been established as a target for cancer drug discovery.[7–9] Via a hydrophobic groove comprised of its Bcl-2 homology (BH) domains BH1, BH2, and BH3, Bcl-x_L binds to the BH3 domains of pro-apoptotic Bcl-2 proteins.[10] This leads to sequestration and thereby functional inhibition of pro-apoptotic Bcl-2 proteins and prevents the induction of apoptosis in tumor cells. Alexidine was found to bind to the BH3 domain binding pocket of Bcl-x_L, and to thereby interfere with protein–protein interactions between Bcl-x_L and the pro-apoptotic proteins Bad and Bak. As a consequence, alexidine induces apoptosis in a number of tumor cell lines derived from the tongue and the pharynx via the intrinsic apoptosis pathway.[6]

Alexidine (**1**) contains two stereogenic centers, but because of its symmetry, only three isomers of alexidine exist: two enantiomers (*R*,*R*)-**1** and (*S*,*S*)-**1**, and the achiral *meso* diastereoisomer (*R*,*S*)-**1** (Fig. 1).

One of the quintessential requirements for chiral drugs admitted for human use today is a solid understanding of the biological effects of the individual enantiomers. In striking contrast to this principle, no studies have been published which analyze the biological effects of the individual chiral isomers of alexidine. All samples of alexidine used either as disinfecting agents or in basic research studies to date consist of the mixture of the two chiral enantiomers (*R*,*R*)-**1** and (*S*,*S*)-**1**, and the achiral isomer (*R*,*S*)-**1**.

Assuming a statistical distribution of the stereogenic centers in the mixture of alexidine isomers, each chiral isomer contributes

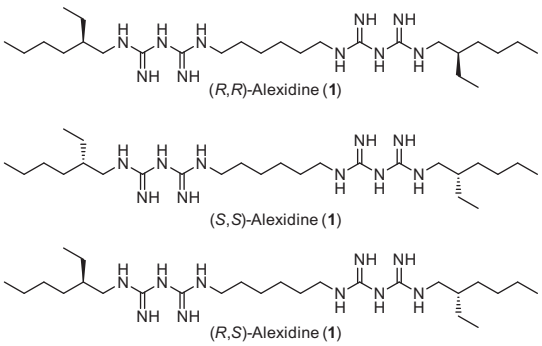

Figure 1. Isomers of alexidine **1**.

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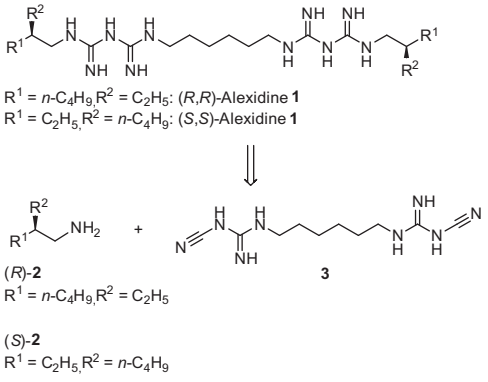


Figure 2. Retrosynthetic approach to alexidine **1**.

25% of the overall mixture, and the achiral isomer contributes 50%. Since all of alexidine's molecular targets reported so far are chiral, it is conceivable that the biological activity of any of the chiral isomers is up to four times higher than the biological activity of the mixture of isomers. This makes it highly desirable to have access to the individual chiral enantiomers of alexidine for biological testing. However, despite alexidine's biological relevance, no synthesis of its enantiomers has been published to date. We therefore sought to develop a synthetic strategy leading to both enantiomers of alexidine in high enantiomeric purity.

Here, we report the first synthesis of highly enantiomerically enriched (*R*,*R*)- and (*S*,*S*)-alexidine **1**. The synthetic strategy can be adapted to the synthesis of enantiomerically enriched alexidine analogs, allowing for the analysis of their structure–activity relationships in biological assays.

2. Results and discussion

2.1. Synthesis

The retrosynthetic analysis (Fig. 2) towards alexidine enantiomers (*R*,*R*)-**1** and (*S*,*S*)-**1** identifies the reaction between 2-ethyl-1-hexylamines (*R*)-**2** and (*S*)-**2**,[11] respectively, and dicyano compound **3** as the key step of the synthesis. Both **2** and **3** can be obtained from readily available starting materials, and are subsequently combined to yield the target molecules (*R*,*R*)-**1** and (*S*,*S*)-**1** in a convergent manner.

The synthetic route towards (2*S*)-ethyl-1-hexylamine (*S*)-**2** started with the published procedure towards the enantiomerically enriched (2*S*)-ethyl-1-hexanol (*S*)-**8**, and is based on pseudoephedrine (*R*,*R*)-**4** (Fig. 3).[12] Synthesis of (2*R*)-ethyl-1-hexanol (*R*)-**8** was carried out in an analogous fashion using the enantiomeric pseudoephedrine (*S*,*S*)-**4**. To this end, pseudoephedrines (*S*,*S*)-**4** and (*R*,*R*)-**4** were acylated with butyric acid anhydride. After deprotonation of the pseudoephedrine amides (*S*,*S*)-**5** and (*R*,*R*)-**5**, they were alkylated with butyl iodide to obtain (*S*,*S*,*R*)-**6** and (*R*,*R*,*S*)-**6**, respectively (Fig. 3). Acid hydrolysis of the chiral auxiliary and lithium aluminium hydride reduction of the liberated acids (*R*)-**7** and (*S*)-**7** provided the alcohols (*R*)-**8** and (*S*)-**8**. The assignment of the absolute configuration of the newly generated stereogenic centers is based on the positive value of the optical rotation of alcohol (*S*)-**8** ($[\alpha]_D^{22} + 2.88, c\ 0.1, CHCl_3$), which is consistent with the assignment of the (*S*)-configuration to the respective 2-ethyl-1-hexanol enantiomer throughout the literature.[11–14]

Alcohols (*R*)-**8** and (*S*)-**8** were converted to N-alkylated phthalimides (*R*)-**9** and (*S*)-**9** under Mitsunobu conditions. Hydrazinolysis of **9** furnished the desired enantiomerically enriched 2-ethylhexyl-1-amines (*R*)-**2** and (*S*)-**2** as their hydrochloride salts. The determination of the optical purities of amines **2** was accomplished by conversion into their corresponding Mosher amides. HPLC analysis of the Mosher amides revealed an enantiomeric excess (ee) of 95.6% for (*R*)-**2**, and 94.8% for (*S*)-**2**. The high enantiomeric purities of amines **2** are consistent with the high enantiomeric excess of 96% reported for generation of the alcohol (*S*)-**8** in the literature,[12] demonstrating that the procedure can also be applied to the generation of the enantiomeric alcohol (*R*)-**8**.

Our synthetic approach to highly enantiomerically pure 2-ethyl-1-hexylamines **2** requires only six synthetic steps and is thus two steps shorter than the previously reported route.[11] In addition, while the reported procedure involves a reductive step

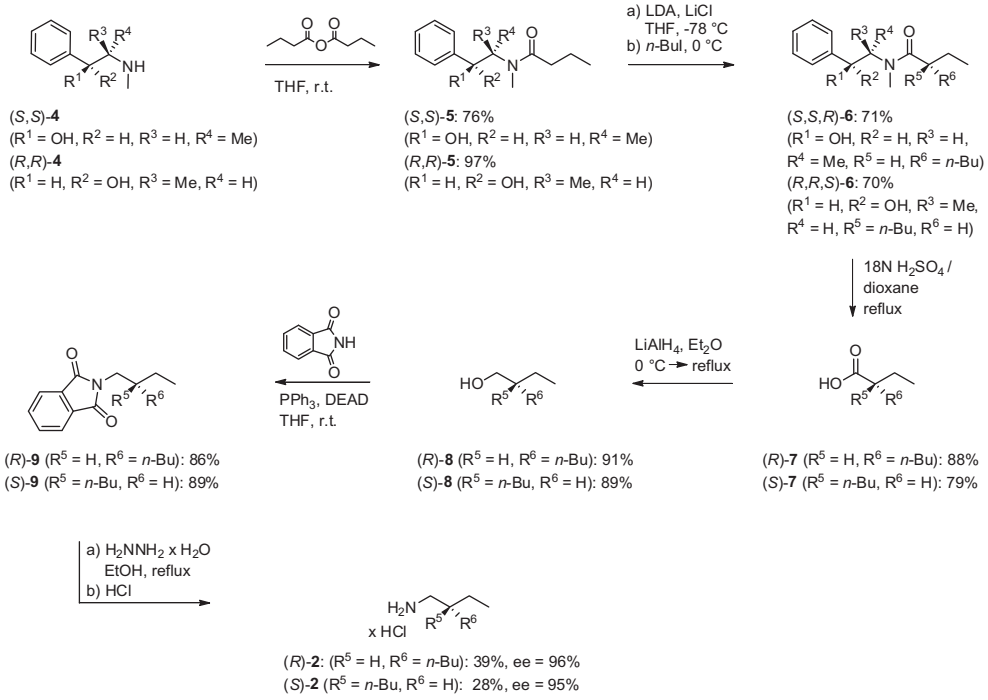


Figure 3. Synthesis of (*R*)- and (*S*)-2-ethyl-1-hexylamines **2**.

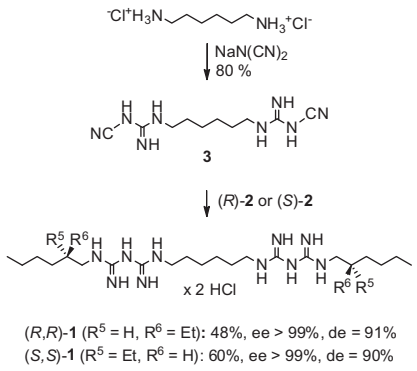


Figure 4. Synthesis of (*R*,*R*)- and (*S*,*S*)-alexidines **1**.

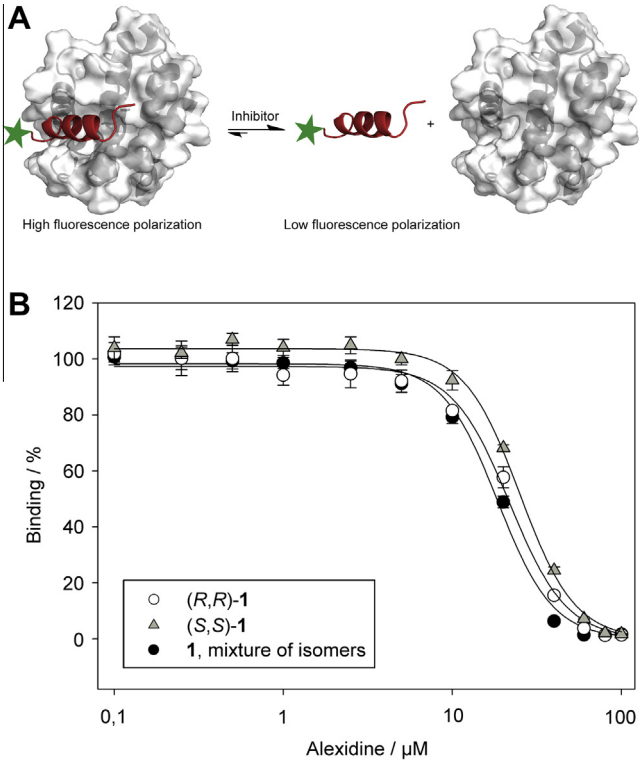


Figure 5. Alexidine inhibits binding between Bcl-x_L and Bak. (A) Principle of the assay. A peptide derived from the Bak BH3 domain (shown in red) is labeled with a fluorophore (shown in green). When bound to Bcl-x_L (shown in light gray), the emitted fluorescence has a high polarization owing to the large molecular weight of the peptide/protein complex. An inhibitor of protein/peptide binding liberates the fluorophore-labeled peptide, leading to a reduction of fluorescence polarization. The structures are based on PDB entry 1BXL.[17] The Figure was generated using PyMol.[18] (B) Activities of (*R*,*R*)- and (*S*,*S*)-alexidine and the commercially available mixture of all three isomers against the interaction between Bcl-x_L and the Bak BH3 domain-derived peptide (5-carboxyfluorescein)-GQVGRQLAIIGDDINR-NH_2[19] as analyzed in fluorescence polarization assays. The mutant Bcl-x_L Δ45–84, in which the flexible loop previously shown to be dispensable for the anti-apoptotic activity of Bcl-x_L is deleted, was used in these assays.[17,20]

using Na–Hg amalgam,[11] the approach described here avoids the use of toxic heavy metals.

Dicyano compound **3** was prepared from 1,6-diaminohexane (used as the dihydrochloride) and sodium dicyanamide.[1] Next, the amines (*R*)-**2** and (*S*)-**2** were reacted separately with **3** following the published procedure[1] to afford the enantiomers of alexidine (Fig. 4). Assuming an unbiased incorporation of the individual enantiomers of 2-ethyl-1-hexylamine **2** into the target compounds **1**, the enantiomeric excess of both (*S*,*S*)-**1** and (*R*,*R*)-**1**

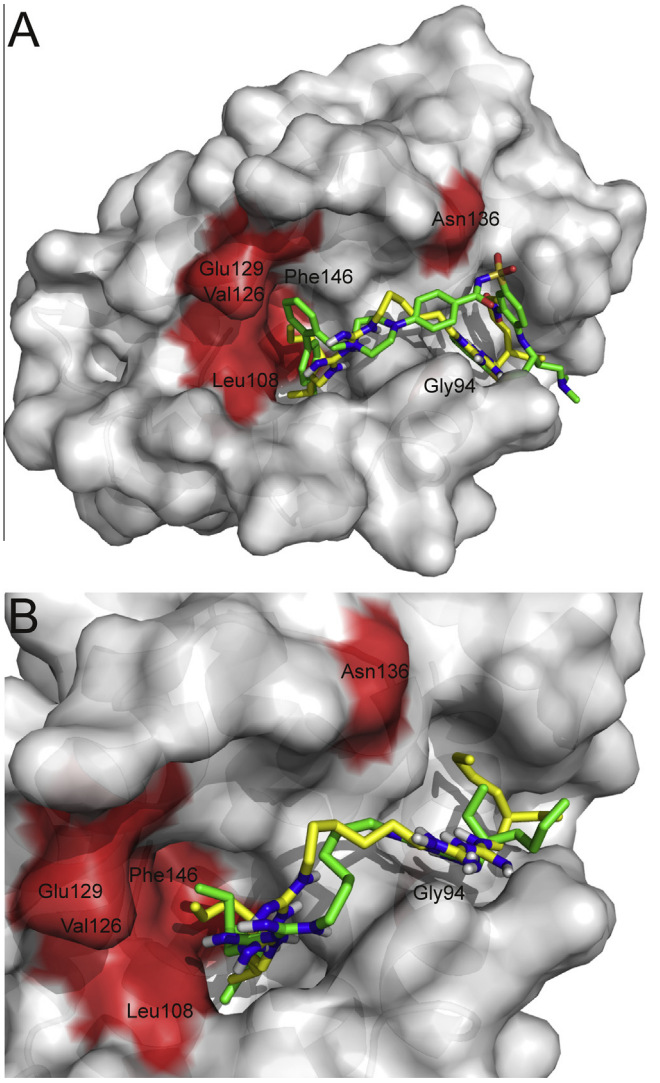


Figure 6. Binding of chiral alexidine isomers to Bcl-x_L. (A) Overlay of the binding mode of ABT-737 (carbon atoms shown in green) to Bcl-x_L according to the co-crystal structure[21] (PDB entry: 2YXJ) and the docking pose of (*R*,*R*)-alexidine **1** (carbon atoms shown in yellow). Amino acids previously shown to display strong, dose-dependent shifts in the ^1H, ^{15}N-HSQC-NMR are marked in red.[6] (B) Overlay of the docking pose of (*R*,*R*)-alexidine **1** (carbon atoms shown in yellow) and (*S*,*S*)-alexidine **1** (carbon atoms shown in green) bound to Bcl-x_L. The Figure was generated using PyMol.[18]

was calculated as exceeding 99.8% (see Supplementary data for calculation of enantiomeric excesses). The increased enantiomeric excesses of the alexidines **1** as compared to the precursor amines **2** is caused by generation of the achiral diastereoisomer (*S*,*R*)-**1**, in the event that one molecule of the respective major enantiomer, and one molecule of the respective minor enantiomer of **2** is incorporated into **1**. Thus, incorporation of one molecule of the respective minor enantiomer of **2** into **1** does not impact the enantiomeric purity, but instead the diastereomeric purity of **1**. This effect has been described in the literature for other compounds which can form achiral *meso*-diastereoisomers.[15,16] Nevertheless, both (*S*,*S*)-**1** and (*R*,*R*)-**1** were still calculated to display high diastereomeric excesses of 90% and 91%, respectively (see Supplementary data for calculation of diastereomeric excesses). Physical separation of the *meso* diastereoisomer by HPLC was found to be not feasible. The optical rotation of the alexidine enantiomers was determined as $[\alpha]_D^{22} + 3.6$ (*c* 0.1 in methanol) for (*R*,*R*)-**1**, and $[\alpha]_D^{22} - 4.3$ (*c* 0.1 in methanol) for (*S*,*S*)-**1**, thus confirming the enantiomeric nature of (*R*,*R*)-**1** and (*S*,*S*)-**1**.

2.2. Biochemical analysis

In order to investigate whether Bcl-x_L can discriminate between the two alexidine enantiomers, we carried out a fluorescence polarization assay. This assay analyzes binding of a fluorophore-labeled peptide derived from the BH3 domain of the pro-apoptotic Bcl-2 protein Bak to the Bcl-x_L protein in the presence of the test compounds (Fig. 5A). Surprisingly, we found that the IC_{50}-values for (*R*,*R*)-alexidine (IC_{50} = 21.3 ± 0.8 μM) and (*S*,*S*)-alexidine (IC_{50} = 25.7 ± 0.5 μM) are not substantially different from one another. They are also very similar to the data obtained with the commercially available mixture of all three alexidine isomers (IC_{50} = 18.3 ± 0.7 μM) (Fig. 5B).[6]

2.3. Molecular docking

To rationalize the results of the fluorescence polarization assay, we carried out molecular docking experiments of the individual alexidine enantiomers against the BH3-domain binding pocket of Bcl-x_L[21] using AutoDock Vina (Fig. 6A).[22] The BH3 binding pocket of Bcl-x_L consists of two subpockets. The narrower subpocket includes residues Leu108, Val126, Glu129, and Phe146. In the crystal structure of Bcl-x_L bound to the high-affinity inhibitor ABT-737,[8] this subpocket is occupied by the 4-chlorobiphenyl moiety of the inhibitor.[21] The wider subpocket comprising Gly94 and Asn136 is filled with the acylsulfonamide part of ABT-737.[21] All of these amino acids have previously been shown to display strong shifts in the ^1H, ^{15}N-HSQC-NMR in the presence of the mixture of alexidine isomers,[6] suggesting that alexidine binds to the BH3 binding pocket of Bcl-x_L. Consistent with the NMR shifts,[6] molecular docking placed (*R*,*R*)-alexidine **1** into the same pocket as ABT-737 (Fig. 6). However, (*S*,*S*)-alexidine **1** fitted equally well into the BH3-domain binding pocket (Fig. 6B). This indicated that both hydrophobic cavities of Bcl-x_L can be occupied by the individual alexidine enantiomers to the same extent owing to the conformational flexibility of the alkyl chains. Therefore, the configuration of the chiral center does not appear to affect the degree of hydrophobic interactions mediated by the 2-ethyl hexyl substituents of (*S*,*S*)- and (*R*,*R*)-alexidine substantially.

2.4. Conclusions

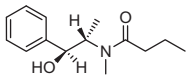
In summary, we have presented the first synthesis of (*S*,*S*)- and (*R*,*R*)-alexidine in high enantiomeric purity. Our approach includes a shorter synthetic route to the building blocks (*S*)- and (*R*)-2-ethyl-1-hexylamine **2** than previously reported,[11] which also does not require the use of toxic heavy metals. The versatility of the pseudoephedrine-based approach to yield α-alkylated alcohols in excellent enantiomeric excesses,[23,24] in combination with their racemization-free conversion under Mitsunobu conditions, will facilitate the generation of diverse chiral primary amines that are suitable for the synthesis of various chiral bisbiguanides. Therefore, the new methodology presented here will allow biological research studies aimed at analyzing the mode of action of chiral bisbiguanides such as alexidine and its analogs.

Even though Bcl-x_L does not appear to discriminate between the individual alexidine enantiomers, it is conceivable that other chiral molecular targets of Bcl-x_L, such as the phospholipids in the bacterial membrane, the mitochondrial phosphatase PTPMT1,[3] or other targets of alexidine to be discovered in the future, may interact substantially different with the individual enantiomers. The synthetic route to highly enantiomerically enriched enantiomers of alexidine presented here will therefore enable future research aimed at understanding the biological activities of alexidine on the molecular level.

3. Experimental

3.1. Synthesis and spectroscopic characterization of compounds 1-3, 5–9

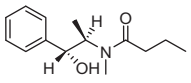
3.1.1. (1*S*,2*S*)-Pseudoephedrine butyramide 5



(*S*,*S*)-**5**

The synthesis was carried out according to the published procedure.[24] Yield: 76%. Melting point: 84–86 °C (Ref.: 82.5–84 °C).[25] ^1H NMR (300 MHz, $CDCl_3$): δ = 0.94 (m, 3H), 1.11 (d, ^3J(H,H) = 7 Hz, 3H), 1.63 (m, 2H), 2.24 (m, 2H), 2.80 (s, 3H), 3.72 (s, 1H), 4.43 (m, 1H), 4.56 (m, 1H), 7.34 (m, 5H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.0, 14.6, 18.5, 36.4, 58.5, 75.6, 76.7, 126.5, 127.7, 128.4, 142.6,175.6 ppm; IR (KBr): $\tilde{\nu}$ = 3289 (s), 3058 (w), 3027 (w), 2993 (w), 2964 (s), 2930 (m), 2876 (m), 1624 (s), 1604 (s), 1454 (s), 1427 (m), 1408 (m), 1378 (w), 1362 (w), 1348 (w), 1320 (m), 1265 (m), 1238 (w), 1203 (w), 1126 (m), 1096 (m), 1051 (s), 921 (w), 906 (w), 790 (w), 767 (s), 706 (s), 607 (m), 516 cm^{-1} (m); ESI-MS $C_{14}H_{21}NO_2$ calcd: 236.2 [M+H$^+$], found: 236.2.

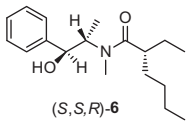
3.1.2. (1*R*,2*R*)-Pseudoephedrine butyramide 5



(*R*,*R*)-**5**

The synthesis was carried out according to the published procedure.[24] Yield: 97%. Melting point: 84–86 °C (Ref.: 85–86.5 °C).[25] ^1H NMR (300 MHz, $CDCl_3$): δ = 0.93 (m, 3H), 1.08 (d, ^3J(H,H) = 7 Hz, 3H), 1.63 (m, 2H), 2.24 (m, 2H), 2.79 (s, 3H), 3.83 (s, 1H), 4.43 (m, 1H), 4.57 (m, 1H), 7.33 (m, 5H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0, 14.6, 18.5, 36.4, 58.5, 75.6, 76.7, 126.5, 127.7, 128.4, 142.6, 175.6 ppm; IR (KBr): $\tilde{\nu}$ = 3290 (s), 3058 (w), 3027 (w), 2993 (w), 2965 (s), 2930 (m), 2876 (m), 1626 (s), 1604 (s), 1454 (s), 1427 (m), 1408 (m), 1379 (w), 1362 (w), 1348 (w), 1320 (m), 1265 (m), 1238 (w), 1203 (w), 1126 (m), 1096 (m), 1051 (s), 921 (w), 906 (w), 790 (w), 767 (s), 706 (s), 607 (m), 516 cm^{-1} (m); ESI-MS $C_{14}H_{21}NO_2$ calcd: 236.2 [M+H$^+$], found: 236.2.

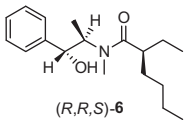
3.1.3. (1*S*,2*S*)-Pseudoephedrine-(2*R*)-ethylhexanamide 6



(*S*,*S*,*R*)-**6**

The synthesis was carried out analogous to the published procedure.[24] Yield: 71%. ^1H NMR (300 MHz, $CDCl_3$): δ = 0.86 (t, ^3J(H,H) = 7 Hz, 6H), 1.06–1.71 (m, 11H), 2.48 (m, 1H), 2.84 (s, 3H), 3.87 (s, 1H), 4.4 (m, 1H), 4.6 (m, 1H), 7.33 (m, 5H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.1, 14.1, 14.7, 23.0, 26.1, 29.8, 32.6, 44.1, 53.5, 58.3, 75.5, 76.5, 126.4, 127.6, 128.4, 142.8,178.9 ppm; IR (Nujol): $\tilde{\nu}$ = 3376 (m), 3062 (w), 3030 (w), 2960 (s), 2930 (s), 2872 (m), 2359 (w), 1617 (s), 1482 (m), 1455 (m), 1411 (w), 1377 (w), 1307 (w), 1258 (w), 1197 (w), 1150 (w), 1112 (m), 1087 (w), 1051 (m), 1028 (w), 763 (w), 737 (w), 701 (m), 608 (w), 518 cm^{-1} (m); ESI-MS $C_{18}H_{29}NO_2$ calcd: 292.2 [M+H$^+$], found: 292.3.

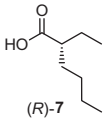
3.1.4. (1*R*,2*R*)-Pseudoephedrine-(2*S*)-ethylhexanamide 6



(*R*,*R*,*S*)-**6**

The synthesis was carried out according to the published procedure.[25] Yield: 70%. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (t, ^3J(H,H) = 7 Hz, 6H), 1.08–1.71 (m, 11H), 2.48 (m, 1H), 2.84 (s, 3H), 3.99 (s, 1H), 4.39 (m, 1H), 4.60 (m, 1H), 7.33 (m, 5H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.1, 14.1, 14.7, 23.0, 26.1, 29.8, 32.6, 44.0, 58.2, 60.5, 75.5, 76.5, 126.4, 127.6, 128.4, 142.8,178.8 ppm; IR (Nujol): $\tilde{\nu}$ = 3375 (m), 3062 (w), 3029 (w), 2960 (s), 2930 (s), 2872 (m), 2360 (w), 1616 (s), 1481 (m), 1456 (m), 1411 (w), 1378 (w), 1309 (w), 1258 (w), 1197 (w), 1151 (w), 1113 (m), 1087 (w), 1051 (m), 1028 (w), 838 (w), 763 (w), 737 (w), 701 (m), 609 (w), 518 cm^{-1} (m); ESI-MS $C_{18}H_{29}NO_2$ calcd: 292.2 [M+H$^+$], found: 292.2.

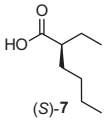
3.1.5. (2*R*)-Ethylhexanoic acid 7



(*R*)-**7**

The synthesis was carried out analogous to the published procedure.[24] Yield: 88%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.89 (t, ^3J(H,H) = 7 Hz, 3H), 0.91 (d, ^3J(H,H) = 7 Hz, 3H), 1.31 (m, 4H), 1.57 (m, 4H), 2.28 (m, 1H), 10.45 (s, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.9, 14.1, 22.8, 25.3, 29.7, 31.6, 47.3, 183.2 ppm; IR (Nujol): $\tilde{\nu}$ = 2963 (s), 2935 (s), 2875 (m), 2863 (m), 1707 (s), 1462 (w), 1417 (w), 1384 (w), 1289 (w), 1272 (w), 1229 (w), 1205 (w), 945 (w), 783 (w), 605 (w), 458 cm^{-1} (w); ESI-MS $C_8H_{16}O_2$ calcd: 143.1 [M−H$^+$], found: 143.0.

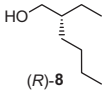
3.1.6. (2*S*)-Ethylhexanoic acid 7



(*S*)-**7**

The synthesis was carried out according to the published procedure.[24] Yield: 79%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.89 (t, ^3J(H,H) = 7 Hz, 3H), 0.94 (d, ^3J(H,H) = 7 Hz, 3H), 1.31 (m, 4H), 1.64 (m, 4H), 2.28 (m, 1H), 10.09 (s, 1H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.9, 14.1, 22.8, 25.3, 29.7, 31.6, 47.2, 183.1 ppm; IR (Nujol): $\tilde{\nu}$ = 2963 (s), 2935 (s), 2875 (m), 2862 (m), 1706 (s), 1460 (m), 1417 (w), 1383 (w), 1289 (w), 1271 (w), 1229 (m), 1205 (w), 1150 (w), 1098 (w), 945 (w), 782 (w), 730 (w), 637 (w), 454 cm^{-1} (w); ESI-MS $C_8H_{16}O_2$ calcd: 143.1 [M+H$^+$], found: 143.1.

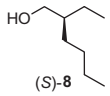
3.1.7. (2*R*)-Ethylhexanol 8



(*R*)-**8**

The synthesis was carried out analogous to the published procedure.[24] Yield: 91%. $[\alpha]_D^{22}$= -2.40 (*c* 0.1 g/100 mL, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.89 (m, 6H), 1.28–1.38 (m, 8H), 1.70 (m, 1H), 3.53 (s, 1H), 3.53 (s, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.2, 14.2, 23.2, 23.5, 29.3, 30.3, 42.1, 65.4 ppm; IR (Nujol): $\tilde{\nu}$ = 3443 (w), 2961 (m), 2925 (m), 2870 (w), 2854 (w), 1634 (m), 1457 (w), 1261 (m), 1096 (m), 1021 (m), 800 (m), 702 (w), 668 (w), 608 (w), 470 cm^{-1} (w). For MS analysis, the alcohol was reacted with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to afford the corresponding Mosher ester. HR-ESI-MS of the Mosher ester: $C_{18}H_{25}F_3O_3$ calcd: 369.1648 [M+Na$^+$], found: 369.1649.

3.1.8. (2*S*)-Ethylhexanol 8



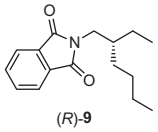
(*S*)-**8**

The synthesis was carried out according to the published procedure.[24] Yield: 89%. $[\alpha]_D^{22}$ + 2.88 (*c* 0.1 g/100 mL, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.9 (m, 6H), 1.3–1.4 (m, 8H), 1.7 (m, 1H), 3.5 (d, ^3J(H,H) = 5 Hz, 2H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.2, 14.2, 23.2, 23.5, 29.3, 30.3, 42.1, 65.4 ppm; IR (Nujol): $\tilde{\nu}$ = 3288 (w), 2959 (s), 2924 (s), 2869 (m), 2853 (m), 1738 (w), 1461 (m), 1377 (m), 1261 (s), 1095 (s), 1021 (s), 974 (w), 863 (w), 801 (s), 702 (w), 662 (w), 606 (w), 491 (w), 456 cm^{-1} (w). For MS analysis, the alcohol was reacted with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to afford the corresponding Mosher ester. HR-ESI-MS of the Mosher ester: $C_{18}H_{25}F_3O_3$ calcd: 369.1648 [M+Na$^+$], found: 369.1649.

3.1.9. Synthesis of 2-(2-ethylhexyl)isoindolin-1,3-diones 9

A solution of 521 mg (4 mmol, 1 equiv) 2-ethylhexanol, 589 mg (4 mmol, 1 equiv) phthalimide and 1.05 g (4 mmol, 1 equiv) triphenylphosphine in 4 ml THF is cooled to 0 °C. To this solution, a solution of 679 mg (4 mmol, 1 equiv) DEAD in 4 ml THF is added and the mixture is stirred at room temperature for 22 h. The solvent is removed in vacuo, the residue suspended in diethylether and filtrated. After evaporation of the solvent in vacuo, the crude product is purified by flash column chromatography (dichloromethane). Racemic **9** has been reported.[26]

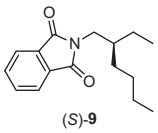
3.1.9.1. 2-((2*R*)-Ethylhexyl)isoindoline-1,3-dione 9



(*R*)-**9**

Yield: 893 mg (86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.91 (m, 6H), 1.28 (m, 8H), 1.83 (m, 1H), 3.56 (s, 1H), 3.58 (s, 1H), 7.71 (m, 2H), 7.82 (m, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 10.6, 14.2, 23.1, 24.0, 28.7, 30.7, 38.5, 42.0, 123.3, 132.3, 134.0, 168.9 ppm; IR (Nujol): $\tilde{\nu}$ = 2959 (s), 2931 (s), 2872 (m), 2860 (m), 1773 (m), 1714 (s), 1614 (w), 1467 (m), 1436 (m), 1397 (s), 1364 (m), 1334 (w), 1188 (w), 1173 (w), 1065 (m), 944 (w), 902 (m), 800 (m), 793 (w), 722 (s), 713 (m), 694 (w), 624 (w), 530 cm^{-1} (w); HR-ESI-MS $C_{16}H_{21}NO_2$ calcd: 260.1645 [M+H$^+$], found: 260.1646.

3.1.9.2. 2-((2*S*)-Ethylhexyl)isoindolin-1,3-dione 9



(*S*)-**9**

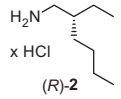
Yield: 925 mg (89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.91 (m, 6H), 1.28 (m, 8H), 1.83 (m, 1H), 3.57 (s, 1H), 3.58 (s, 1H), 7.7 (m,

2H), 7.8 (m, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 10.6, 14.2, 23.1, 24.0, 28.7, 30.7, 38.5, 42.1, 123.3, 132.3, 134.0, 168.9 ppm. IR (Nujol): $\tilde{\nu}$ = 2953 (s), 2922 (s), 2850 (s), 1718 (m), 1699 (w), 1683 (w), 1646 (w), 1507 (w), 1457 (m), 1419 (w), 1396 (w), 1376 (w), 1365 (w), 1113 (w), 779 (w), 721 (w), 640 (w), 601 (w), 456 cm^{-1} (w); HR-ESI-MS $C_{16}H_{21}NO_2$ calcd: 260.1645 [M+H$^+$], found: 260.1647.

3.1.10. Synthesis of 2-ethylhexylamine hydrochlorides 2

A solution of 778 mg (3.0 mmol, 1 equiv) 2-(2-ethylhexyl)isoindolin-1,3-dione **5** and 150 mg (3.0 mmol, 1 equiv) hydrazine monohydrate in 12 ml ethanol is heated to reflux for 15 min. The resulting suspension is filtrated, the filtrate acidified with 5 mL of 6 M hydrochloric acid, and washed with diethylether. The aqueous phase is made alkaline by addition of 2 M NaOH and extracted with diethylether. The organic phases are combined, dried with magnesium sulfate and filtrated. Four milliliter of 6 M hydrochloric acid is added and the solvent removed in vacuo to afford the pure amine as the hydrochloric salt.

3.1.10.1. (2*R*)-Ethylhexylamine hydrochloride 2

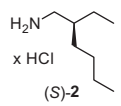


(*R*)-**2**

Yield: 194.0 mg (39%, 95% ee). Melting point: 80 °C; $[\alpha]_D^{22}$ + 1.5 (*c* 0.1 g/100 mL, CH_3OH). ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 0.82 (m, 6H), 1.25 (m, 8H), 1.57 (m, 1H), 2.65 (m, 2H), 8.11 (s, 3H) ppm; ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 10.2, 13.9, 22.3, 22.8, 28.0, 29.5, 36.9, 41.5, ppm; IR (Nujol): $\tilde{\nu}$ = 3442 (s), 3019 (s), 2961 (s), 2929 (s), 2874 (s), 2863 (s), 2665 (w), 1717 (m), 1661 (m), 1601 (w), 1495 (m), 1466 (m), 1396 (w), 1380 (w), 1349 (w), 1330 (w), 1306 (w), 1263 (w), 1171 (w), 1082 (w), 1023 (w), 791 (w), 782 (w), 725 (w), 628 (w), 494 (w), 477 cm^{-1} (w); ESI-MS $C_8H_{19}N$ calcd: 130.2 [M+H$^+$], found: 130.2.

In order to determine the enantiomeric excess of (2*R*)-ethylhexylamine hydrochloride **2**, 10 mg (0.08 mmol) were dissolved in 1 ml dichloromethane and cooled to 0 °C. Triethylamine (18 mg, 0.18 mmol) and (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (22 mg, 0.09 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, water and diethyl ether were added. The aqueous phase was extracted three times with diethyl ether. Subsequently, the organic phase was dried over sodium sulfate and evaporated under reduced pressure. The Mosher amide was analyzed using HPLC to determine the enantiomeric excess of the amine (Chiralcel® OJ column, 4.6 mm × 25 cm). Ee = 95%; retention time: 16.6 min (corresponding to the Mosher amide of (*S*)-**2** as the minor isomer) and 19.5 min (corresponding to the Mosher amide of (*R*)-**2** as the major isomer).

3.1.10.2. (2*S*)-Ethylhexylamine hydrochloride 2

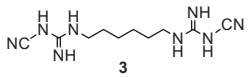


(*S*)-**2**

Yield: 141.0 mg (28%, 95% ee). Melting point: 83 °C; $[\alpha]_D^{22}$ − 4.4 (*c* 0.1 g/100 mL, CH_3OH). ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 0.82 (m, 6H), 1.24 (m, 8H), 1.57 (m, 1H), 2.66 (m, 2H), 8.05 (s, 3H) ppm; ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 10.2, 14.0, 22.4, 22.8, 27.9, 29.5, 36.9, 41.6, ppm; IR (Nujol): $\tilde{\nu}$ = 3443 (s), 3022 (s), 2960 (s), 2929 (s), 2874 (s), 2360 (w), 1648 (m), 1636 (m), 1603 (m), 1558 (w), 1496 (m), 1465 (m), 1458 (m), 1380 (w), 1331 (w), 1082 (w), 792 (w), 683 (w), 628 (w), 563 cm^{-1} (w); ESI-MS $C_8H_{19}N$ calcd: 130.2 [M+H$^+$], found: 130.3.

Determination of the enantiomeric excess of (2*S*)-ethylhexylamine hydrochloride **2**, was carried out analogous to its enantiomer (*R*)-**2** by conversion of the amine to the Mosher amide. This was analyzed using HPLC (Chiralcel® OJ column, 4.6 mm × 25 cm). Ee = 95%; Retention time: 16.3 min (corresponding to the Mosher amide of (*S*)-**2** as the major isomer) and 19.1 min (corresponding to the Mosher amide of (*R*)-**2** as the minor isomer).

3.1.11. 1,6-Di-(N^3-cyano-N^1-guanidino)hexane 3

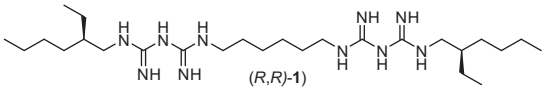

3

Compound **3** was synthesized according to the published procedure.[1] In brief, a solution of 3.78 g (20.0 mmol, 1 equiv) 1,6-hexamethylenediamine dihydrochloride and 3.56 g (40.0 mmol, 2 equiv) sodium dicyanamide in 28 mL *n*-butanol was heated to reflux for 8 h. After cooling to room temperature, the solid was filtered off and washed with butanol and cold water. Recrystallization from water afforded pure compound **3**. Yield: 80%. Melting point: 195 °C (Ref.: 202–203 °C).[1] ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 1.23 (m, 4H), 1.39 (m, 4H), 3.02 (m, 4H), 6.63–6.83 (m, 6H) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 25.9, 28.9, 40.8, 118.4, 161.2 ppm; IR (KBr): $\tilde{\nu}$ = 3372 (s), 3159 (s), 2940 (s), 2940 (m), 2857 (m), 2172 (s), 1650 (s), 1625 (s), 1475 (m), 1439 (s), 1383 (m), 1322 (m), 1128 (m), 928 (w), 733 (w), 689 (w), 561 (m), 541 cm^{-1} (m); ESI-MS $C_{10}H_{18}N_8$ calcd: 249.2 [M−H$^+$], found: 249.3.

3.1.12. Synthesis of 1,6-bis[5-(2-ethylhexyl)biguanido]hexane dihydrochlorides 1

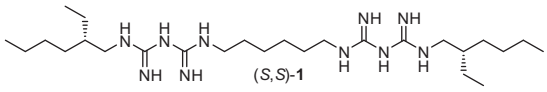
63.0 mg (0.25 mmol, 1 equiv) 1,6-di-(N^3-cyano-N^1-guanidino)-hexane **3** and 82.9 mg (0.5 mmol, 2 equiv) (*S*)-2-ethylhexylamine hydrochloride **2** (for synthesis of (*S*,*S*)-**1**) or (*R*)-2-ethylhexylamine hydrochloride (*R*)-**2** (for synthesis of (*R*,*R*)-**1**) were thoroughly mixed and heated to 155 °C for 2 h. After completion of the reaction, the crude product was recrystallized from ethanol–ether.

3.1.12.1. (*R*,*R*)-Alexidine 1


(*R*,*R*)-**1**)

Yield: 70.0 mg (48%). Melting point: 208 °C (Ref.: 219–221 °C);[27] Ee >99%; de = 91%; $[\alpha]_D^{22}$ + 3.6 (*c* 0.1 g/100 mL, CH_3OH). ^{1}H NMR (300 MHz, CD_3OD): δ = 0.94 (m, 12H), 1.34–1.42 (m, 18H), 1.60 (m, 6H), 3.25 (m, 8H) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ = 11.3, 14.5, 24.1, 25.2, 27.7, 30.0, 30.4, 32.0, 40.9, 42.5, 45.4, 160.4, 160.5 ppm; IR (KBr): $\tilde{\nu}$ = 3424 (s), 2925 (m), 2855 (m), 2340 (w), 2134 (w), 2025 (w), 1632 (m), 1548 (m), 1462 (m), 1384 (m), 1165 (m), 1112 (m), 593 (w), 551 (m), 473 cm^{-1} (m); HR-ESI-MS $C_{26}H_{56}N_{10}$ calcd: 509.4762 [M+H$^+$], found: 509.4765.

3.1.12.2. (*S,S*)-Alexidine 1



Yield: 87.0 mg (60%). Melting point: 221 °C (Ref.: 219–221 °C);[27] Ee >99%; de = 90%; $[\alpha]_D^{22}$ − 4.3 (*c* 0.1 g/100 mL, CH_3OH). ^1H NMR (400 MHz, CD_3OD): δ = 0.92 (m, 12H), 1.32–1.40 (m, 18H), 1.58 (m, 6H), 3.19 (m, 4H), 3.23 (m, 4H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ = 11.3, 14.5, 24.1, 25.2, 27.7, 30.1, 30.6, 32.0, 40.8, 42.5, 45.4, 160.1, 160.2 ppm; IR (KBr): $\tilde{\nu}$ = 3313 (m), 2959 (m), 2928 (m), 2871 (m), 2341 (w), 2130 (w), 2025 (w), 1632 (s), 1547 (s), 1462 (m), 1384 (m), 1166 (m), 1032 (m), 668 (w), 591 (w), 550 (w), 411 cm^{-1} (m); HR-ESI-MS $C_{26}H_{56}N_{10}$ calcd: 509.4762 [M+H$^+$], found: 509.4767.

3.2. Fluorescence polarization assay

The effect of the test compounds on binding between Bcl-x_L and a 5-carboxyfluorescein-labeled Bak-derived peptide was essentially carried out as described.[6] In brief, Bcl-x_L (amino acids 1–209, Δ45–84) cloned into pET29, a kind gift from Professor Ho Sup Yoon (Nanyang Technical University, Singapore),[17] was expressed from *Escherichia coli* Rosetta (Novagen) as previously described,[28] purified by affinity chromatography, and extensively dialyzed against buffer (50 mM Hepes, 100 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet-40 substitute, pH 7.5). Binding of this protein to the Bak BH3-derived peptide (5-carboxyfluorescein)-GQVGRQLAIIGDDINR-NH_2[19] was analyzed by fluorescence polarization. Peptide and protein stocks were diluted in 10 mM Tris, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% DMSO, pH 8.0. Final concentrations in the assay buffer: 5-carboxyfluorescein-labeled peptide: 10 nM; Bcl-x_L (amino acids 1–209, Δ45–84): 40 nM. Fluorescence polarization was read in an Infinite 500 plate reader (Tecan). Assays were carried out in black 384-well plates (Corning no. 3573). Inhibition was calculated based on curve fits using SigmaPlot (SPSS Science Software). The mixture of alexidine isomers (as dihydrochloride, CAS# 1715-30-6) used in the fluorescence polarization assay was purchased from Toronto Research Chemicals and purified by reversed phase chromatography at the Core Facility of the Max-Planck Institute of Biochemistry.

3.3. Molecular docking

Docking experiments were performed with AutoDock Vina.[22] Default settings were used with the exception of the exhaustiveness, which was set to 50. Docking experiments were run using the Bcl-x_L structure from PDB entry 2YXJ after removal of the ligand ABT-737. The docking poses calculated by AutoDock Vina were visualized using PyMOL.[18]

Acknowledgments

We would like to thank Prof. Ho Sup Yoon (Nanyang Technical University, Singapore) for the Bcl-x_L expression plasmid. We extend our thanks to the core facility of the Max Planck Institute of Biochemistry, Martinsried, for experimental support, and to Angela Hollis for critical reading of the manuscript. This study was supported by the University of Leipzig.

Supplementary data

Supplementary data associated (^1H and ^{13}C NMR spectra of the new compounds, calculation of enantiomeric and diastereomeric excesses of alexidines) with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.057.

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