Cycloserine Fatty Acid Derivatives as Prodrugs: Synthesis, Degradation and *in Vitro* Skin Permeability

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Various 4,5-dihydroisoxazol-3-yl fatty acid ester derivatives of cycloserine were synthesized to improve skin permeation of cycloserine. The ester derivatives were prepared by using the *tert*-butoxycarbonyl (*t*-Boc) protection strategy. The 4,5-dihydroisoxazol-3-yl esters were readily hydrolysed in an aqueous buffer solution, and the degradation profiles showed both specific acid and specific base catalysis. In 50% human serum the formation of cycloserine was observed, but enzymatic catalysis was limited. Delivery through hairless mouse skin was investigated, and the apparent permeability coefficient was measured based on the flux of cycloserine into the receptor phase. The skin permeation of cycloserine across the hairless mouse skin was increased up to 20-fold by the fatty acid esters. The 4,5-dihydroisoxazol-3-yl fatty acid esters of cycloserine can therefore be considered as new topical prodrugs with the potential use in treatment of various skin infections.

Key words prodrug; cycloserine; permeability; hydrolysis; hairless mouse skin

Cycloserine (Seromycin, 4(*R*)-amino-3-isoxazolidinone) is a well-known drug used for the treatment of tuberculosis and certain genitourinary infections, especially when caused by Enterobacteria or *E. coli*.[1)] The drug was first isolated and tested[2)] in 1955, but for the past few years it has also been used in clinical trials against Alzheimer disease and to increase learning capabilities in animals.[3)] Systemic side effects restrict the usage of cycloserine and topical application for local delivery has limited value because cycloserine is a highly water-soluble compound and poorly permeable through lipophilic membranes such as the skin. Only a few prodrugs of cycloserine have previously been synthesized for various purposes, such as improving biological effects,[4)] preventing dimerization in water,[5)] enhancing delivery through biological membranes[6)] and for possible use as anticancer drugs.[7)] These prodrugs were based on chemical modification of the nitrogen atoms. In the present study we have synthesized fatty acid derivatives on the 3-oxo group yielding a more lipophilic prodrugs. Previously, we have synthesized fatty acid derivatives of metronidazole, and we were able to achieve a 40-fold increase in the delivery of the drug through hairless mouse skin.[8)] In this case, the fatty acids were used as transport moieties to achieve increased absorption of the drug. An additional benefit of the use of fatty acids as transport moieties is that fatty acids can act as penetration enhancers, and the prodrug can therefore facilitate its own absorption. Furthermore, fatty acids can also have some biological activity of their own, especially antimicrobial activity.[9)] Using a fatty acid as a transport moiety can therefore be expected to potentiate the activity of an antimicrobial drug.

In the present study we report investigations of 4,5-dihydroisoxazol-3-yl fatty acid esters of cycloserine, as possible prodrugs to enhance dermal delivery of cycloserine. Systematic side effects of the oral drug treatment of certain skin infections could therefore be avoided by topical application with cycloserine prodrugs. For example *Mycobacterium avium* skin infection in the dermis, which is currently treated[10)] by surgery, followed by antibiotic treatment with combination of cycloserine, isoniazid and clarithromycin.

Results and Discussion

All the compounds were synthesized according to the general procedure described in Chart 1. The amino function of cycloserine was protected with *tert*-butoxycarbonyl (*t*-Boc) (60% yields), and the 4,5-dihydroisoxazol-3-yl esters of acetic acid, octanoic acid, lauric acid and oleic acid were obtained by the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) coupling strategy or reaction with the corresponding acetic anhydride (47—93% yields), followed by removal of the protection group with trifluoroacetic acid (TFA) (69—81% yields). The prodrug structures were confirmed by ^{1}H- and ^{13}C-NMR.

Cycloserine (**1**) is relatively stable in the pH range investigated. No degradation at 60 °C was observed over 24 h (Table 1). All the protected compounds **3**—**6** showed similar pH/chemical stability dependence with specific base catalysis above pH 5 and the maximum stability between pH 3 and 5 (Fig. 1). The pH rate profiles for compounds **7**—**10** were similar. The ester derivatives were formed on an enol form of the relatively acid 3-isoxazolidone (pK_a=4.4).[1)] The hydrolysis of the prodrugs was therefore rapid (Fig. 2). In the case of cycloserine laurate the neutral hydrolysis was about 10 times

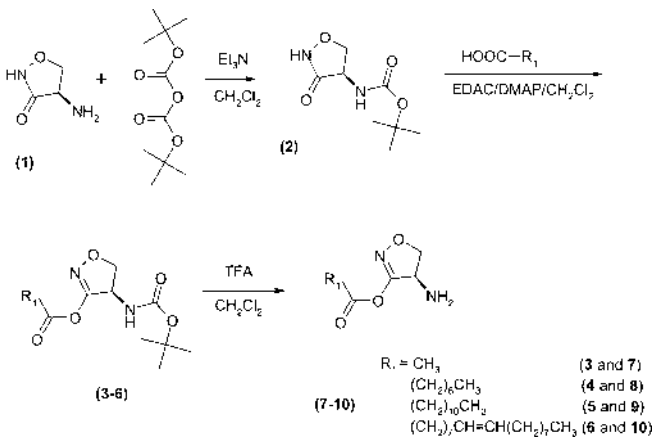

Chart 1. Total Synthesis of the Prodrugs

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Table 1. Rate of Degradation (Mean±S.E.; $n=3$) of Compounds **2**—**10** in Aqueous Buffers (pH 2.0, pH 7.0) at 60 °C

Compounds	$k_{obs.}$ (h^{-1}) pH 7.0	$k_{obs.}$ (h^{-1}) pH 2.0
1	No degr.	No degr.
2	Not determ.	0.82±0.14
3	12.2±1.1	2.69±0.01
4	15.2±1.2	2.08±0.08
5	1.44±0.28	0.42±0.05
6	1.03±0.32	Not determ.
7	1.06±0.26	0.82±0.14
8	1.33±0.39	0.93±0.09
9	4.26±1.30	1.43±0.21
10	3.99±1.62	1.25±0.12

Table 2. The Apparent Permeability Coefficients (Mean±S.E.; $n=3$) and Calculated log P Values for Compounds **1**—**10**

Compounds	MW (g/mol)	Estimated[a] log P	Permeability (cm/h)×10^3
1	102	−1.7	0.5±0.4
2	202	−0.3	Not determ.
3	244	0.5	1.3±0.6
4	328	3.3	5.5±2.7
5	400	4.9	9.0±1.7
6	466	7.1	11.9±4.4
7	144	−0.8	1.7±0.5
8	228	1.9	3.5±1.1
9	284	3.6	4.5±1.1
10	366	5.7	6.2±1.6

a) log P calculated with CS Chemdraw Ultra from CambridgeSoft.

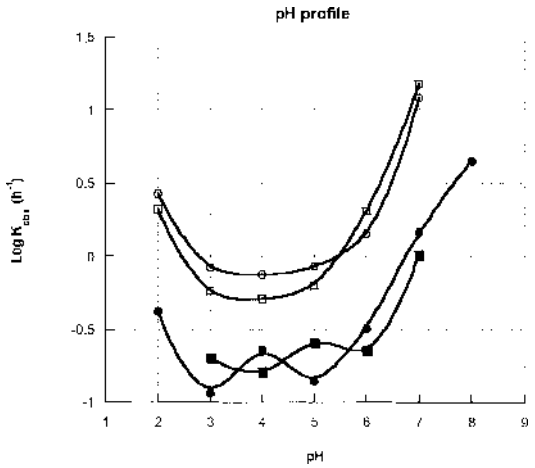

Fig. 1. The pH-Degradation Rate Profile of Compounds **3** (○), **4** (□), **5** (●) and **6** (■) at 60 °C

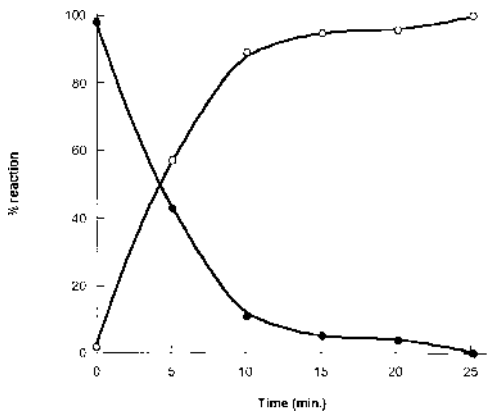

Fig. 2. Degradation of Prodrug **10** (●) and Formation of Cycloserine (○) in Buffer Solution (pH 8.0) at 60 °C

faster than the hydrolysis of the prodrug metronidazolyl laurate.[8)]

The enzymatic hydrolysis of the prodrugs by bovine serum esterase was investigated. In the presence of bovine serum esterase there was no increase in the hydrolysis rate relative to chemical hydrolysis in buffer solutions. However, 50% human serum did have some catalytic effect, with a 14% increase ($k_{obs.}=0.133\ h^{-1}$) in the formation of cycloserine from compound **10** relative to the reference solution ($k_{obs.}=0.116\ h^{-1}$). Thus, it could be concluded that the fatty acid esters of the electron-deficient 4,5-dihydroisoxazoline ring system are poor substrates for hydrolytic enzymes. In contrast, there was more than a 100% increase in the degradation rate of metronidazolyl fatty acid esters in the presence of human serum.[8)]

Compound **1** had activity against *Staph. aureus* with minimum inhibition concentration (MIC)=32 μg/ml and minimum lethal concentration (MLC)=64 μg/ml, and there was also activity against *E. coli* (MIC=16 μg/ml). Anti-bacterial activity was not present in the derivatives (MIC>500 μg/ml). Thus, the derivatives could be considered true prodrugs, lacking biological activity of the parent compound.

When the prodrugs where applied to hairless mouse skin in a propylene glycol donor phase the parent drug appeared in the receptor phase. The cycloserine prodrugs were rather unstable and therefore three types of mechanism could be considered: degradation of the prodrug in the donor phase and subsequent permeation of the parent drug through the skin; permeation of the prodrug through the skin and degradation in the receptor phase; or degradation of the prodrug within skin tissue. The first mechanism could not explain the presence of cycloserine in the receptor phase as no degradation of prodrug in the donor phase was observed after 48 h. The second mechanism is unlikely as no trace of prodrug (less than 0, 1 μg/ml) could be measured in the receptor phase. Therefore it was assumed that the main mechanism was degradation of prodrug within the skin. The apparent permeation coefficient was calculated on the basis of flux of cycloserine into the receptor phase. The presence or absence of the *t*-Boc protection group did not have a significant effect on permeation through the skin. Thus, the compounds with the protection group could also be considered as prodrugs for dermal delivery. The permeability coefficient increased 2 to 20-fold, relative to compound **1** (Table 2). The enhanced permeability correlated with increasing lipophilicity (calculated log P values), whereas the differences in degradation rates (Table 1) where much smaller and did not correlate with the permeability differences. When the prodrugs degraded within the skin the fatty acid component, which has absorption enhancing properties, was released. The absorption enhancing effects of the free fatty acids could also have contributed to the fact that the permeation was higher with fatty acid derived prodrugs (compounds **4**—**6**, **8**—**10**) than with acetic acid derived prodrugs (compounds **3**, **7**). However a larger study will be required to quantify the contribution of this absorption enhancement.

Conclusion

The present study describes the synthesis of 4,5-dihydroisoxazol-3-yl fatty acid esters of cycloserine. These ester derivatives are prodrugs that can improve cycloserine skin permeation up to 20-fold as compared to the unmodified parent drug. These prodrugs can therefore be considered for local delivery of cycloserine, especially for the treatment of skin infections. Thus the systematic side effects of cycloserine could be avoided.

Experimental

General Methods ^{1}H- and ^{13}C-NMR spectra were measured on either a 400 MHz Bruker AM 400 spectrometer or a 250 MHz Bruker AC 250 P NMR spectrometer with tetramethylsilane (Me_4Si) as an internal reference and $CDCl_3$ or D_2O as a solvent. Both ^{1}H- and ^{13}C-NMR spectral data are reported in parts per million (δ) relative to Me_4Si.

(**1**) 4-Amino-3-isoxazolidone (Cycloserine): ^{1}H-NMR (D_2O) δ: 4.54 (dd, 1H, J=8, 8 Hz, CH_AH), 4.32 (dd, 1H, J=8, 8 Hz, CH), 4.22 (dd, 1H, J=8, 8 Hz, CHH_B). ^{13}C-NMR (D_2O) δ: 173.8 (C=O), 73.5 (CH), 57.5 (CH_2).

(**2**) *N-tert*-Butoxycabonyl-4-amino-3-isoxazolidone (*t*-Boc-Cycloserine): 2.00 g (19 mmol) of compound **1** dissolved in 50 ml of 50% aq. tetrahydrofuran (THF), 2.10 g (20 mmol) of triethylamine added to the solution. 4.10 g (19 mmol) of di-*tert*-butyldicarbonate dissolved in 15 ml of THF was added dropwise to the solution over a period of 1 h and stirred for 5 h at room temperature. After removal of the solvent in vacuum, the compound was purified by silica gel column chromatography. Elution with ethyl acetate/acetone mixture (4 : 1) gave 2.30 g of white crystals (58% yield). mp 134—136 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.37 (bs, 1H, NH), 4.76 (t, 1H, J=8 Hz, CH_AH), 4.65 (bs, 1H, CH), 4.10 (t, 1H, J=8 Hz, CHH_B), 1.49 (s, 9H, CH_3). ^{13}C-NMR ($CDCl_3$) δ: 171.4 (C=O), 156.4 (C=O), 79.8 (C), 75.5 (CH), 53.5 (CH_2), 26.3 ($(CH_3)_3$).

(**3**) *N-tert*-Butoxycarbonyl-4-amino-4,5-dihydroisoxazol-3-yl Acetate (*t*-Boc-Cycloserine-Acetate): 2.01 g (10 mmol) of compound **2**, 1.30 g (13 mmol) of acetic acid anhydride and 100 mg of 4-(dimethylamino)pyridine (DMAP) was dissolved in 30 ml of CH_2Cl_2 and stirred at room temperature overnight. The solution was evaporated, and the crude was purified by silica gel chromatography. Elution with ethyl acetate afforded 1.94 g of white crystals (79% yield); mp 86—89 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.34 (bs, 1H), 4.69 (bs, 2H), 4.12 (dd, 1H, J=15, 12 Hz), 2.39 (s, 3H), 1.39 (s, 9H). ^{13}C-NMR ($CDCl_3$) δ: 166.7, 163.4, 155.0, 81.1, 71.6, 53.5, 28.1, 22.9.

General Procedure for Compounds 4—6 Compound **2** (between 5 and 10 mmol) and the carboxylic acid were dissolved in 25 ml of CH_2Cl_2 in the presence of 10% excess of EDAC and 50 mg of DMAP and the solution was refluxed overnight. The solution was then washed 3× with 25 ml 5% $NaHCO_3$, and the solvent was evaporated *in vacuo* and the crude product was purified by silica gel chromatography. Finally the compound was dried under reduced pressure (0.02 mbar) to obtain solid material.

(**4**) *N-tert*-Butoxycarbonyl-4-amino-4,5-dihydroisoxazol-3-yl Octanoate (*t*-Boc-Cycloserine-Octanoate): (93% yield); mp 66—68 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.48 (bs, 1H), 4.67 (bs, 2H), 4.13 (dd, 1H, J=10, 8 Hz), 2.68 (t, 2H, J=7.5 Hz), 1.60 (m, 2H), 1.38 (s, 8H), 1.22 (s, 9H), 0.82 (t, 3H, J=3.75 Hz). ^{13}C-NMR ($CDCl_3$) δ: 166.8, 166.5, 155.0, 80.8, 71.4, 53.5, 28.0, 35.1, 31.4, 28.8, 28.7, 23.7, 22.3, 13.8.

(**5**) *N-tert*-Butoxycarbonyl-4-amino-4,5-dihydroisoxazol-3-yl Laurate (*t*-Boc-Cycloserine-Laurate): White crystals (82% yield); mp 26—30 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.13 (bs, 1H), 4.82 (t, 1H, J=8 Hz), 4.74 (bs, 1H), 4.15 (dd, 1H, J=11, 8 Hz), 2.78 (t, 2H, J=Hz), 1.68 (m, 2H), 1.46 (s, 9H), 1.26 (s, 16H), 0.88 (t, 3H, J=8 Hz). ^{13}C-NMR ($CDCl_3$) δ: 166.8, 166.4, 155.0, 80.9, 71.6, 53.6, 28.1, 35.3, 31.8, 29.6, 29.4, 29.1, 29.0, 28.8, 27.0, 23.8, 22.5, 14.0.

(**6**) *N-tert*-Butoxycarbonyl-4-amino-4,5-dihydroisoxazol-3-yl Oleate (*t*-Boc-Cycloserine-Oleate): White crystals (47% yield); mp 36—39 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.33 (bs, 1H), 4.77 (bs, 2H), 4.16 (dd, 1H, J=12, 12 Hz), 2.76 (t, 2H, J=7.5 Hz), 2.01, 1.99 (2s, 4H), 1.66 (m, 2H), 1.45 (s, 9H), 1.26 (bs, 20H), 0.87 (t, 3H, J=7.5 Hz). ^{13}C-NMR ($CDCl_3$) δ: 166.8, 166.4, 155.0, 129.9, 129.6, 81.0, 71.8, 53.6, 28.1, 35.3, 31.8, 29.6, 29.4, 29.2, 29.1, 29.0, 28.9, 28.8, 27.1, 27.0, 23.8 , 22.5, 14.0.

General Procedure for Compounds 7—10 One gram of the parent compound with the *t*-Boc protection group was dissolved in 5 ml of CH_2Cl_2. Then 3 ml of TFA was added to the solution and stirred at room temperature for 1 h. The solution was evaporated, and the crude product was purified by silica gel chromatography.

(**7**) 4-Amino-4,5-dihydroisoxazol-3-yl Acetate (Cycloserine Acetate): Clear oil (74% yield). ^{1}H-NMR (DMSO-d_6) δ: 6.74 (bs, 2H), 3.93 (bs, 2H), 3.75 (m, 1H), 2.39 (s, 3H). ^{13}C-NMR (DMSO-d_6) δ: 171.8, 153.5, 64.4, 51.6, 22.8.

(**8**) 4-Amino-4,5-dihydroisoxazol-3-yl Octate (Cycloserine Octanoate): Yellowish oil (81% yield). ^{1}H-NMR (DMSO-d_6) δ: 6.75 (bs, 1H), 3.92 (bs, 2H), 3.76 (m, 1H), 2.00 (m, 2H), 1.48 (m, 2H), 1.24 (s, 8H), 0.86 (t, 3H, J=3.75 Hz). ^{13}C-NMR (DMSO-d_6) δ: 171.7, 153.5, 64.5, 51.7, 35.1, 31.3, 28.9, 28.6, 23.5, 22.3, 13.8.

(**9**) 4-Amino-4,5-dihydroisoxazol-3-yl Laurate (Cycloserine Laurate): White crystals (69% yield); mp 38—41 °C. ^{1}H-NMR (DMSO-d_6) δ: 4.07 (bs, 1H), 3.37 (bs, 4H), 1.50 (m, 2H), 1.20 (bs, 16H), 0.85 (t, 3H, J=7.5 Hz). ^{13}C-NMR (DMSO-d_6) δ: 171.6, 153.4, 64.6, 51.7, 31-22, 13.9.

(**10**) 4-Amino-4,5-dihydroisoxazol-3-yl Oleate (Cycloserine Oleate): White crystals (76% yield); mp 36—39 °C. ^{1}H-NMR (DMSO-d_6) δ: 5.31 (bs, 2H), 4.17 (bs, 2H), 3.76 (s, 1H), 3.43 (bs, 2H), 1.98, 1.97 (2s, 4H), 1.50 (m, 2H), 1.24 (bs, 20H), 0.85 (t, 3H, J=7.5 Hz). ^{13}C-NMR (DMSO-d_6) δ: 167.3, 164.7, 129.5, 72.8, 53.0, 40-22, 13.8.

HPLC Measurements The HPLC system consisted of a Merck Hitachi L-7100 gradient solvent delivery system with a L-7400 UV detector. For all sample separations a Waters reverse-phase C8 column (150×4.6 mm, 5 μm) was used. The detection was done at a 230-nm wavelength, and the flow rate was 1.0 ml/min. The mobile phases consisted of MeOH and 0.015% octansulfonic acid in H_2O. The volume fractions and retention times for each compound were as follows: (**1**) 10 (MeOH) : 90 (0.015% octansulfonic acid) (250 mm column), 3.0 min; (**2**) (15 : 85), 3.2 min; (**3**) (60 : 40), 3.2 min; (**4**) (60 : 40), 3.0 min; (**5**) (65 : 35), 2.8 min; (**6**) (70 : 30), 3.0 min; (**7**) (34 : 66), 3.1 min; (**8**) (40 : 60), 2.9 min; (**9**) (60 : 40), 3.8 min; (**10**) (65 : 35), 3.3 min.

Degradation Rate Studies The degradation rates of the compounds were determined in Theorell–Stenhagen buffer systems[11] (acetate, phosphate, borate, NaOH; pH values from 2 to 10) at 60 °C. The rate constants ($k_{obs.}$) were obtained by linear regression of the logarithm of HPLC peak intensity. The enzymatic degradation was studied with porcine liver esterase (Sigma numbers E-2884) in a 20 mM phosphate buffer (pH 7.4) at 37 °C. A blank medium with no enzyme was used as a control. Degradation in 50% human serum was studied. A blank medium with no human serum was used as a control.

Antibacterial Test The antibacterial tests were performed at the Icelandic National Hospital, Department of Microbiology. The bacteria in a growth media were exposed to diluted solutions of the compounds; the MIC and MLC were measured according to the dilution series and expressed in μg/ml of compound needed. The reaction was incubated for 24 h at 37 °C. This method was developed by NCCLS (National Committee for Clinical Laboratory Standards).[12] The bacterial strains, *Enterococcus faecalis* (ATCC 29212), *Staph. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were chosen because they are the most common cause of infection at hospitals. ATCC is American Type Culture Collection of standard bacteria strains.

Permeation Studies Female hairless mice, (3CH/Tif h/h) obtained from Bommice (Denmark) were sacrificed by cervical dislocation and their full-thickness skins were removed. The outer surface of the skin was rinsed with 35% (v/v) methanol in water and subsequently with distilled water to remove any contamination. The skin was placed in Franz diffusion cells of type FDC 400 15 FF (Vangard International Inc., U.S.A.). The receiver compartment had a volume of 12.3 ml. The surface area of the skin in the diffusion cell was 1.77 cm^2. The receptor phase consisted of phosphate buffer saline pH 7.4 (Ph.Eur., 2nd Ed., VII.1.3.) containing 0.3% (w/v) Brij-58 to ensure sufficient drug solubility in the receptor phase. The receptor phase was sonicated under vacuum prior to usage to remove dissolved air. The skin diffusion cells were stirred with a magnetic bar and kept at 37 °C by circulating water through an external jacket. The donor phase consisted of a 1 mg/ml solution of the prodrug in propylene glycol, which also prevent degradations during the experiment. The solution in the donor chamber was analyzed to determine degradation after 48 h. 2 ml of the donor phase were applied to the skin surface and the donor chamber covered with parafilm. Samples (100 μl) of receptor phase were removed from the cells at various time intervals of up to 48 h and replaced with a fresh buffer solution. The samples were kept frozen until analysed by HPLC. The apparent permeability coefficient was calculated from the flux of cycloserine into the receptor phase divided by the concentration of the prodrug in the donor phase.

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