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Novel cyclosporin derivatives featuring enhanced skin penetration despite increased molecular weight

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Abstract—Topical cyclosporin A (CsA, **1**) is not effective in the treatment of skin diseases, due to its low skin penetration. Following a prodrug strategy, a series of novel derivatives of **1** and of 2-[*O*-(2-hydroxyethyl)-D-Ser[8]]-CsA (SDZ IMM 125, **5**) with potentially enhanced skin penetration properties were synthesized, in order to achieve higher levels of the active parent drugs in the skin. Permeation through skin and prodrug/drug levels in the skin were measured in vitro using rat and human skin. Introduction of a polar side chain, either in the form of a positively charged quaternary amine, a negatively charged phosphate or sulfate, or an amphiphilic phosphocholine moiety, generally increased permeability. Maximal increase in permeability through skin relative to CsA was up to 300-fold with rat skin, and up to 16-fold with human skin. Penetration into skin, as evaluated by measurement of prodrug/drug concentrations in the skin after 48 h, could be enhanced up to 14-fold (rat and human skin). Increases of permeation rates and skin concentrations showed no strict correlation. Using the phosphate **10** as prodrug, a 2.5-fold higher concentration of the active parent compound (**5**) could be achieved in rat skin as when administering **5** itself. The results demonstrate that in contrast with the '500 Dalton rule', which postulates poor skin penetration of molecules larger than 500 Da, high skin permeation can be achieved also with compounds of a molecular weight in the range between 1200 and 1600 Da. Results also indicate that in principle higher skin levels of active drug can be attained with a prodrug strategy in this class of compounds.

1. Introduction

The immunosuppressant cyclosporin A (CsA) is well established as a therapeutic option in a large variety of skin diseases,[1] including psoriasis,[2] atopic dermatitis,[3] and allergic contact dermatitis.[4] Systemic treatment, however, may be associated with side effects such as nephrotoxicity and hypertension. Topical use of CsA would minimize the risk of such side effects, and thus extend its use to mild and moderate forms of the skin diseases. However, trials to treat skin diseases topically with a variety of CsA formulations have failed,[5] with no or only marginal efficacy being reported (with the exception of one uncontrolled and so far unconfirmed study[6]). However, beneficial effects were reported in patients with plaque psoriasis upon intralesional injection of CsA.[7] These findings indicate that the lack of topical efficacy might be due to insufficient penetration of the drug into the skin.[8] In fact, it has been argued that the molecular weight of a compound must be under 500 Da to allow sufficient penetration into the deeper layers of the skin, while larger molecules would not be able to pass the corneal layer.[9]

However, quantitative structure activity relationships within series of skin permeants indicate that skin permeation depends not only on molecular weight, but also on other physicochemical properties (such as charge, and lipophilicity as measured by the octanol/water partition coefficient) and structural descriptors have to be integrated in order to predict skin permeation.[10] Furthermore, it has been shown that addition of certain fatty acids and long-chain alcohols to solutions of CsA significantly increased its skin penetration and that this enhancement is dependent on structural properties of the excipients.[11] This stimulated us to synthesize a series of cyclosporin derivatives with modified

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✠ This manuscript is dedicated to the memory of Dr. Hermann Vyplel, who died together with his family in a tragic airplane accident.

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physicochemical properties, in particular polar charged side chains, in order to study the influence of the structural modifications on skin penetration. The ultimate aim was to identify prodrugs, which would allow enhanced drug concentrations in the skin after topical administration. After topical application to rat and human skin in vitro, the diffusion of the compounds into the skin (penetration) and through the skin (permeation into the receptor fluid of the diffusion chambers) was assessed, and the release of parent drug was monitored. We identified compounds, which show greatly enhanced penetration and permeation as compared to CsA, although featuring increased molecular weight relative to the parent compounds.

2. Chemistry

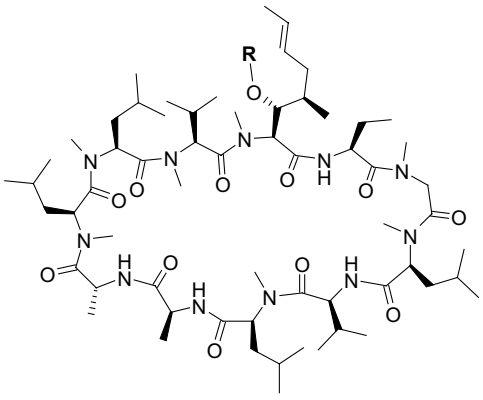
Two derivatives of CsA (**1**) with a positively charged side chain (Table 1) were prepared by the reaction of (*O*-chloroacetyl)MeBmt[1]-cyclosporin[12] (**2**) with dimethylamine and, respectively, of CsA with γ-dimethylaminobutyric acid in the presence of dicyclo-hexylcarbodiimide/4-dimethylaminopyridine; the corresponding quaternary ammonium compounds **3** and **4** were obtained upon reaction with an excess of iodomethane followed by exchange of iodide to chloride as a pharmaceutically more acceptable salt form. The yield in the esterification step with γ-dimethylaminobutyric acid to steric hindrance at the secondary hydroxyl group of the MeBmt-side chain.

2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (SDZ IMM 125, **5**), a biologically highly active CsA derivative[13,14] with a chemically more easily accessible primary hydroxyl group, was selected as starting material for further investigations. Reaction of **5** with 11-dimethylamino-undecanoic acid in the presence of dicyclohexylcarbodiimide/4-dimethylaminopyridine and subsequent quaternization of the amino group with an excess of iodomethane yielded the ω-trimethylammonioalkanoic ester **6** (Scheme 1).

For the preparation of the carbonate **7** and the carbamate **8**, 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**) was treated with phosgene in dichloromethane in the presence of lutidine and the resulting chlorocarbonate was reacted without isolation with the appropriate ω-dimethylamino-alkanol or -alkylamine. Subsequent quaternization afforded the end products (Scheme 1). Derivative **9** featuring a carboxylic acid at the side chain was prepared by reaction of **5** with succinic acid anhydride in acetone in the presence of 4-dimethylaminopyridine.

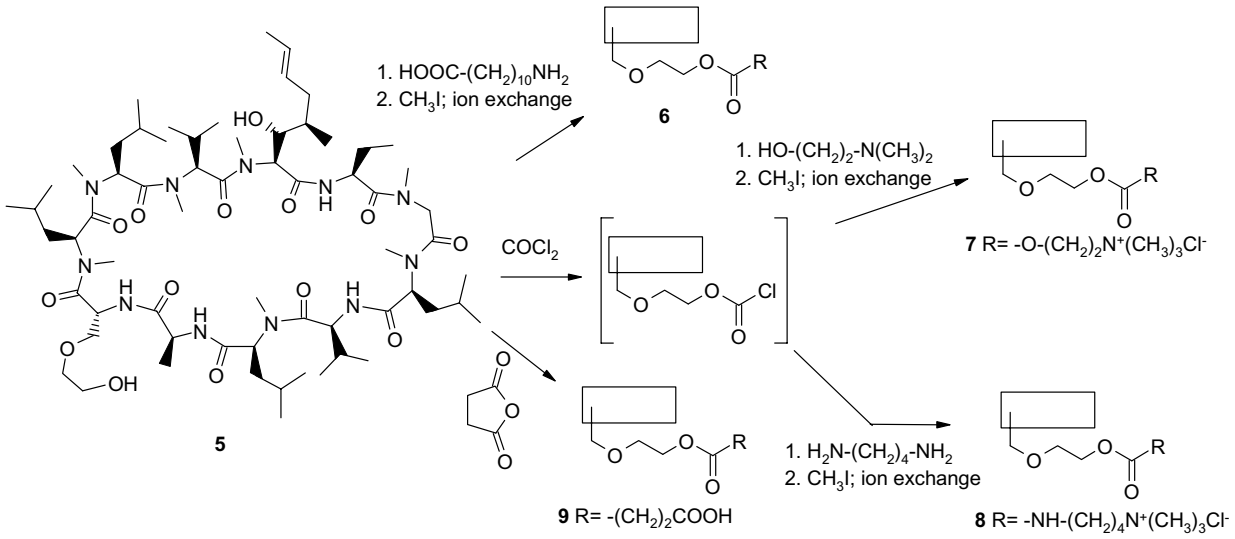
Preparation of derivatives of 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**) with phosphate-containing side chains is shown in Scheme 2. Treating **5** with phosphoryl chloride in trimethyl phosphate in the presence of lutidine, afforded phosphate **10** in good yield after aqueous work-up.[15] Similarly, the monoethyl phosphate **11** was obtained by quenching with one equivalent of ethanol instead of water. Treatment with dimethylamino-ethanol, however, failed to give the desired dimethylamino-ethyl phosphate. Therefore, **5** was converted with freshly prepared 2-bromoethylphosphorodichloridate,[15] into the bromoethyl phosphate which reacted cleanly with trimethylamine[16] to give the phosphocholine derivative **12**. Preparation of the sulfate derivative **13** was accomplished by treating D-Ser[8]-cyclosporin [17] with sodium hydride and cyclic glycolsulfate in tetrahydrofurane in 85% yield (Scheme 3).

Table 1. Penetration/permeation of CsA (**1**) and two positively charged derivatives acylated at the hydroxy group of MeBmt[1] into/through rat skin in vitro



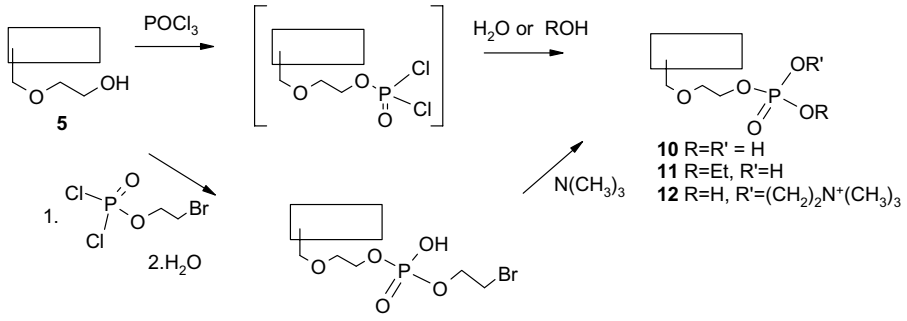
No.	R	Skin concentration[a] [mg/g]		Permeation rate[a] [μg/cm^2/h]	
		Prodrug	1	Prodrug	1
1	H	—	0.70 ± 0.10	—	0.07 ± 0.01
3	$-COCH_2N^+(CH_3)_3Cl^-$	0.96 ± 0.12	n.d.[b]	8.2 ± 1.1	n.d.
4	$-CO(CH_2)_3N^+(CH_3)_3Cl^-$	0.99 ± 0.08	n.d.	14.4 ± 1.2	n.d.

[a] Data represent means ± standard deviation of triplicate determinations.
[b] n.d., not detectable, the limit of quantification being approximately 2 μg/g for skin, and 0.005 μg/cm^2/h for permeation.

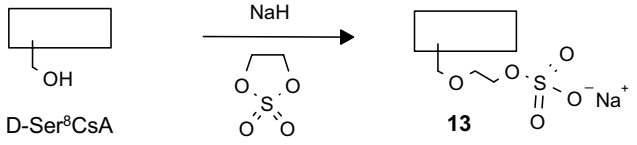


The boxes depict the peptidic ring of **5**, the substituent being attached at the α-carbon of D-Ser[8]

Scheme 1. Synthesis of derivatives of 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**).



Scheme 2. Synthesis of derivatives of 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**) with phosphate-containing side chains.



Scheme 3. Synthesis of a sulfate derivative (**13**) of 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**).

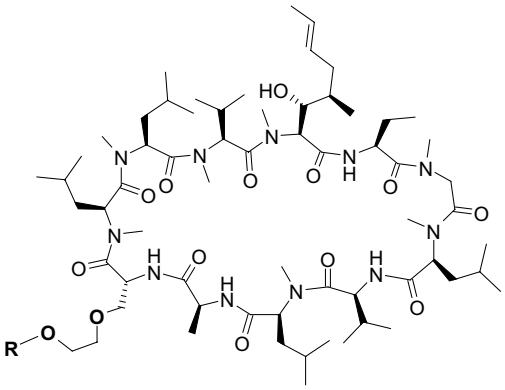
3. Results

Skin penetration was assessed using rat or human skin mounted in Franz-type diffusion cells.[18] Following epicutaneous application of 1% solutions, drug permeation into the receptor fluid of the diffusion chamber was measured and the permeation rate was calculated; at the end of the 48-h experiments, drug levels in the skin were determined after removal of the stratum corneum by tape stripping. Concentrations of prodrug and parent drug were measured in parallel.

As expected from the results of previous studies,[11] cyclosporin A (**1**) exhibited a low permeation through rat skin in vitro (permeation rate: 70 ng/cm^2/h, Table 1). Introduction of the polar trimethylammonioacetyl group at the MeBmt[1] hydroxy group (compound **3**) resulted in an increase of the permeation rate by a factor of 117 as compared to **1**. Extension of the alkyl chain between the ester function and the trimethylammonio group (compound **4**) led to a further enhancement of the permeation (factor of about 200 relative to **1**). While permeation of **3** and **4** was greatly enhanced, skin concentrations were only marginally higher than with **1**. Derivatives of CsA acylated at the MeBmt[1] hydroxyl group have very low, if any, immunosuppressive activity;[19] therefore, for both, **3** and **4**, cleavage by an esterase to yield active **1** is required for biological activity. However, concentrations of **1** were below 5% of total compound, both in the receptor and in the skin, indicating poor cleavage of the respective ester bonds. This result, together with the low reactivity of the hydroxy group at MeBmt[1] observed in acylation reactions (see chemistry section), suggested that this site is too hindered for cleavage by esterases.

2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA (**5**) showed low penetration/permeation, similar to **1** (Table 2). Introduction of an ω-trimethylammonioalkanoyl moiety at the primary hydroxy group (compound **6**) resulted in an

Table 2. Penetration/permeation of *O*-hydroxyethyl-D-Ser[8]CsA (**5**) and derivatives into/through rat skin in vitro



No.	R	Skin concentration[a] [mg/g]		Permeation rate[a] [μg/cm^2/h]	
		Prodrug	5	Prodrug	5
5	H	—	0.77 ± 0.15	—	0.34 ± 0.08
6	$-CO(CH_2)_{10}N^+(CH_3)_3Cl^-$	3.3 ± 0.3	0.49 ± 0.06	0.7 ± 0.1	0.22 ± 0.03
7	$-COO(CH_2)_2N^+(CH_3)_3Cl^-$	10.9 ± 0.9	0.12 ± 0.02	22.3 ± 3.1	0.05 ± 0.01
8	$-CONH(CH_2)_4N^+(CH_3)_3Cl^-$	1.2 ± 0.1	n.d.	11.6 ± 1.1	n.d.
9	$-CO(CH_2)_2COOH$	4.3 ± 0.2	n.d.	0.87 ± 0.12	n.d.
10	$-P(O)(OH)_2$	0.6 ± 0.1	1.98 ± 0.09	8.4 ± 0.9	6.4 ± 0.8
11	$-P(O)OC_2H_5(OH)$	0.9 ± 0.2	0.38 ± 0.02	10.7 ± 0.9	0.18 ± 0.02
12	$-P(O)(O^-)(O(CH_2)_2N^+(CH_3)_3)$	0.6 ± 0.1	n.d.	12.7 ± 1.2	n.d.
13	$-OSO_2ONa$	0.7 ± 0.15	n.d.	19.4 ± 1.5	n.d.

[a] Data represent means ± standard deviation of triplicate determinations.

increase of permeation through rat skin by a factor of 2. Compound **6** was partially cleaved in the skin to yield **5**, about 13% of total drug measured in skin being in the form of the parent drug (**5**) (Table 2). This was also reflected by a time-dependent increase of **5** in the receptor fluid (no cleavage of the prodrugs occurred in the receptor fluid in the absence of skin). In summary, however, concentration of **5** in the skin after application of the prodrug remained below that attained with **5** itself.

In compounds **7** and **8**, positively charged side chains are attached to **5** via a carbonate and carbamate linkage, respectively. With the carbonate **7**, greatly increased penetration and permeation of the prodrug into/through rat skin was observed, but only a low degree of conversion to **5** (Table 2). The carbamate **8** showed enhanced permeation, a small increase of skin concentration, but no conversion to the parent drug **5**.

In the succinyl monoester **9** a negatively charged side chain is introduced in **5**. While featuring enhanced penetration and permeation, cleavage of **9** to **5** was undetectable. In contrast, with the phosphate monoester **10**, a drug/prodrug ratio of 3.3:1 was observed in the skin, and the drug concentration in skin was 2.5 times higher than after administration of **5** itself. The phosphodiester **11** showed lower penetration and permeation as compared to **10** and also lower conversion to **5**. Introduction of a phosphorylcholine group (compound **12**) resulted in markedly enhanced permeation. However, skin concentration remained low and no conversion to the parent compound was observed. Similarly, the sulfate **13**, while readily permeating through the skin, featured low skin levels and was not converted to the parent drug.

A selection of compounds was also tested with human skin. As expected from previous experience[20] skin concentrations and permeation rates of **1** and **5** were much lower (up to a factor of 25) than with rat skin (Table 3). Compound **4** showed greatly enhanced permeation (factor of about 50 compared to **1**). In contrast to the findings in rat skin, also the concentration of prodrug in the skin was considerably higher (factor 13) than that achieved with **1**. Cleavage of **4** to the parent compound was, however, undetectable, as with rat skin.

The 2-[*O*-(hydroxyethyl)-D-Ser[8]]-CsA derivative **6** was selected for evaluation due to the high concentrations of the prodrug achieved in rat skin. In fact, concentrations of **6** were 10-fold higher than those achieved with **5** in human skin. However, very poor cleavage of **6** to **5** was observed (drug/prodrug ratio 0.02:1). Likewise, with the carbonate **7** increased penetration/permeation was found, but no detectable cleavage to the parent drug. Finally, also the phosphate **10** showed enhanced uptake into human skin and increased passage into the receptor fluid (factors of about 3 and 10 as compared to **5**, respectively). However, unlike with rat skin, there occurred only marginal conversion of **10** to **5** in human skin.

4. Discussion

Although oral CsA is highly effective, topical treatment of patients with inflammatory skin diseases with CsA

Table 3. Penetration/permeation of test compounds into/through human skin in vitro

No.	Skin concentration[a] [mg/g]		Permeation rate[a] [μg/cm^2/h]	
	Prodrug	Parent	Prodrug	Parent
1	—	0.065 ± 0.006	—	0.023 ± 0.007
4	0.87 ± 0.09	n.d.	1.08 ± 0.03	n.d.
5	—	0.078 ± 0.02	—	0.031 ± 0.005
6	0.79 ± 0.19	0.017 0.04	0.18 ± 0.03	0.009 ± 0.002
7	0.16 ± 0.02	n.d.	0.29 ± 0.03	n.d.
10	0.22 ± 0.03	0.003 ± 0.001	0.37 ± 0.05	n.d.

[a] Data represent means ± standard deviation of triplicate determinations.

failed to show satisfactory results, most probably due to insufficient drug penetration into skin. Numerous attempts to solve the problem by optimized galenical formulations were unsuccessful.[5,11] The preparation of prodrugs with improved skin penetration properties, which can be cleaved in the skin to the active drug, appeared to be a promising alternative approach. However, the '500 Dalton rule',[9] postulating that an effective topical drug in dermatology should have a molecular weight below 500 Da, argues against this strategy, since the already high molecular weight of CsA (1203 Da) would necessarily be further increased in a prodrug. We now found that by introducing polar side chains in CsA or SDZ IMM 125, permeability can be enhanced substantially, the positive effect induced by structural modification clearly overcompensating the influence of the molecular weight increase. With the CsA derivative **4** (molecular weight: 1330 Da), for example, permeability was enhanced by a factor of about 200 with rat skin and a factor of about 50 with human skin. Likewise, some derivatives of SDZ IMM 125 (**5**) with either positively or negatively charged side chains showed up to about 60-fold increased permeability through rat skin relative to the parent drug and up to 300-fold increase relative to CsA (see compounds **7** and **13**, Table 2). With human skin, the maximal increase in permeability of an SDZ IMM 125 derivative was 16-fold. Notably, the permeation rates through human skin achieved with some of the derivatives (compounds **4**, **6**, **7**, and **10)** are in the same order of magnitude as those of topical corticosteroids used in dermatological practice, when measured under identical experimental conditions.[21] The observed permeation enhancement is in line with a report that attachment of arginine oligomers to CsA facilitates topical delivery and anti-inflammatory activity in an animal model of skin disease.[22] Furthermore, the calcineurin inhibitors pimecrolimus and tacrolimus (molecular weight 810 and 804 Da, respectively) have proven clinical efficacy in atopic dermatitis patients after topical administration, as well as in psoriasis patients after application to the face or intertriginous areas.[23] Thus, it is obvious that the '500 Dalton rule' is not generally applicable.

From our data it is apparent that introduction of a polar side chain increases skin penetration properties of the cyclosporins, either in the form of a positively charged quaternary amine (**3**, **4**, **6, 7**), negatively charged carboxylic (**9**), phosphate (**10**, **11**), or sulfate (**13**) groups, or an amphiphilic phosphocholine moiety (**12**). Addition of a side chain with a carboxylic group (**9**) was effective as well.

For the topical treatment of skin disorders it is essential that the drug attains sufficiently high concentrations in the skin, ideally without permeating further into the circulation from where it would be distributed to other compartments of the body and cause unwanted systemic side effects. The targets of CsA are inflammatory T effector cells that accumulate in the dermis and in the deeper layers of the epidermis in skin lesions. While useful algorithms for prediction of skin permeation exist,[10] no theoretical models have been established, that would allow to predict skin concentrations. While skin permeation is mainly governed by the partitioning of a compound between formulation, stratum corneum (as the major diffusion barrier) and receptor fluid, retention of a compound in the skin is likely to be influenced in addition by interaction between the drug and constituents of the skin (cellular membranes, proteins). In the present series of compounds, it is apparent that skin permeation and penetration (i.e., levels within the skin) do not vary in parallel. The maximal increase in skin concentration of prodrug over parent drug was about 14-fold with both rat and human skin.

A further issue is the need for prodrug cleavage within the skin. While it is long known that mammalian skin contains unspecific esterase and acidic and alkaline phosphatase activity,[24] any detailed characterization of the substrate requirements of these enzymes is not available. For most of our ester and phosphate type prodrugs only a low degree of conversion to the parent compounds within rat skin was observed. The exception is the phosphate **10** for which the 3.3:1 drug/prodrug ratio indicates substantial cleavage in the skin. Together with the observed enhanced permeation rate this results in about 2.5-fold higher levels of parent drug in rat skin than when applying the latter directly. Conversion of **10** in human skin was, however, much lower, illustrating the existence of species differences in the metabolic activity of the skin. The carbamate- (**8**), sulfate- (**13**) and phosphocholine-derivative (**12**) and the ester **9** were not cleaved, presumably due to the high substrate specificity of the esterases, sulfatases, and sphingomyelinases.[24,25]

In conclusion, these results demonstrate that it is possible to design derivatives of cyclosporins with high permeability and increased skin penetration, despite their high molecular weight, thus challenging the '500 Dalton rule'. Results also indicate that levels of active compound in the skin can be enhanced in principle after topical administration, using a prodrug strategy in this class of high molecular weight compounds.

5. Experimental

5.1. General procedures

The NMR experiments were recorded at 300 K on a Bruker AVANCE 500 MHz spectrometer equipped with a 5 mm inverse broadband probehead with z-gradient, operating at 500.13 MHz for ^{1}H, 125.76 MHz for ^{13}C, and 202.46 MHz for ^{31}P. ^{1}H and ^{13}C signal assignments were derived from ^{1}H, ^{13}C-jmod, gs-COSY, gs-HSQC, gs-HMBC, and ^{31}P experiments.[26] Chemical shifts are given in parts per million (ppm) and referenced to tetramethylsilane ($\delta = 0.00$ ppm) for ^{1}H, residual $CDCl_3$ solvent signals ($\delta = 77.0$ ppm) for ^{13}C, and 80% H_3PO_4 in D_2O (external standard, $\delta = 0.0$ ppm) for ^{31}P. Due to the restriction to constitution analysis and strong signal overlap, centered ^{1}H chemical shifts are listed without coupling constant values. In order to facilitate the signal assignment inscriptions, the numbering refers to Figure 1: the C atoms of the amino-acid residues (Arabic numbers 1 to 11) are marked with Greek letters, starting from the backbone; C atoms of sub-residues are marked with additional superscripted Roman letters. In all cases, signal assignment was performed only for the major conformer; in cases where the signal set of a second conformer was visible in the ^{1}H spectrum, its relative concentration was less than 10%. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics 9.4 Tesla APEX-III FT-MS spectrometer in electrospray mode (+/− ions) with flow injection. Optical rotations were determined with a Perkin–Elmer 141 polarimeter at 20 °C. Analytical thin-layer chromatography was performed on Silica Gel 60 F254 glass plates (Merck) with visualization by immersion in a bath containing H_2SO_4 concd. (35 mL), acetic acid (20 mL), $Bi(NO_3)_3$ (1.7 g), and KI (40%, 100 mL) in water (1000 mL), and subsequent drying. Preparative column chromatography was performed on Merck–Lichroprep columns (silica gel, 40–63 μm) under pressure (0.2 mPa). Solvents were AR-grade and were used without purification. Unless noted otherwise, all reagents were obtained from commercial suppliers and were used without purification. The starting materials CsA (**1**), [*O*-(chloroacetyl) MeBmt^{1}]-cyclosporin (**2**),[12] D-Ser^{8}]-cyclosporin,[17] and **5** (2-[*O*-(hydroxyethyl)-D-Ser^{8}]-CsA)[14] were provided by Novartis Pharma Basel.

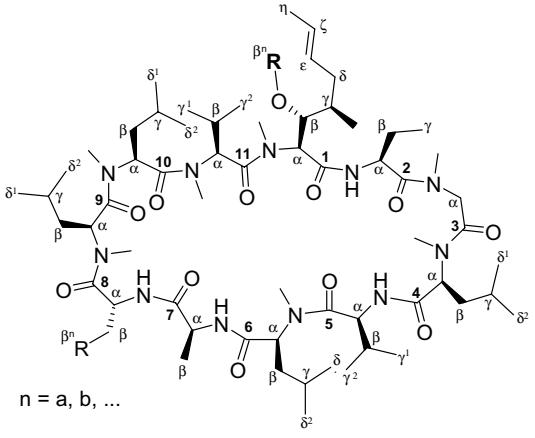


Figure 1. Annotation of residues in NMR spectra.

5.2. [*O*-(Trimethylammonio-acetyl)MeBmt^{1}]-cyclosporin chloride (3)

[*O*-(Chloroacetyl) MeBmt^{1}]-cyclosporin (**2**, 3.6 g, 2.8 mmol) was dissolved in a solution of dimethylamine in ethanol (5.6 M, 50 mL) and stirred overnight at room temperature. After evaporation, the residue was chromatographed twice over silica gel (ethyl acetate/acetone/*n*-hexane 1:1:1 and CH_2Cl_2/CH_3OH 96:4) affording 1.56 g (42%) of a white foam of [(*O*-dimethylammonio-acetyl)MeBmt^{1}]-cyclosporin. The compound (1.29 g, 1 mmol) was dissolved in acetone (50 mL) and methyl iodide (1.42 g, 10 mmol) was added with stirring at room temperature. After 1 h the reaction mixture was evaporated and the residue was dissolved in ethyl ether (50 mL). The ether mixture was stirred overnight at room temperature. The precipitate was filtered off and washed with ether. The product was dissolved in THF/water 1:1 and passed through an Amberlite IRA 401 S (chloride form) column to yield 0.77 g (59%) of **3**. Mp = 175–178 °C; TLC: $R_f = 0.14$ ($CH_2Cl_2/CH_3OH/H_2O$/AcOH 80:8:1:1); ^{1}H NMR ($CDCl_3$): 5.63 (1α); 5.66 (1β); 1.90 (1γ); 0.92 (1γ-Me); 2.06+1.68 (1δ); 5.02 (1ε); 5.45 (1ζ); 1.59 (1η); 3.21 (1-NMe); 5.04+4.26 (1β^{b}); 3.73 (1β^{b}-NMe$_3$); 4.92 (2α); 1.69 (2β); 0.86 (2γ); 8.60 (2-NH); 4.65+3.18 (3α); 3.46 (3-NMe); 5.36 (4α); 1.97+1.63 (4β); 1.43 (4γ); 0.94 (4δ^{1}); 0.87 (4δ^{2}); 3.07 (4-NMe); 4.79 (5α); 2.40 (5β); 1.01 (5γ^{1}); 0.79 (5γ^{2}); 7.45 (5-NH); 5.19 (6α); 2.19+1.07 (6β); 1.93 (6γ); 1.04 (6δ^{1}); 0.69 (6δ^{2}); 3.21 (6-NMe); 4.39 (7α); 1.26 (7β); 7.97 (7-NH); 4.85 (8α); 1.26 (8β); 7.59 (8-NH); 5.63 (9α); 2.27+1.10 (9β); 1.31 (9γ); 0.95 (9δ^{1}); 0.82 (9δ^{2}); 3.19 (9-NMe); 5.20 (10α); 1.68+1.47 (10β); 1.45 (10γ); 1.16 (10δ^{1}); 0.95 (10δ^{2}); 2.61 (10-NMe); 4.61 (11α); 2.18 (11β); 0.92 (11γ^{1}); 0.90 (11γ^{2}); 2.68 (11-NMe). ^{13}C NMR: 167.35 (1); 55.36 (1α); 76.66 (1β); 32.90 (1γ); 17.87 (1γ-Me); 33.53 (1δ); 127.66 (1ε); 127.97 (1ζ); 17.62 (1η); 32.30 (1-NMe); 164.35 (1β^{a}); 63.23 (1β^{b}); 54.46 (1β^{b}-NMe$_3$); 172.62 (2); 48.91 (2α); 24.87 (2β); 9.83 (2γ); 170.78 (3); 50.15 (3α); 39.31 (3-NMe); 169.75 (4); 55.26 (4α); 35.78 (4β); 24.74 (4γ); 24.66 (4δ^{1}); 21.24 (4δ^{2}); 31.26 (4-NMe); 173.71 (5); 54.66 (5α); 31.78 (5β); 19.55 (5γ^{1}); 18.15 (5γ^{2}); 171.20 (6); 54.40 (6α); 36.97 (6β); 24.38 (6γ); 24.60 (6δ^{1}); 21.12 (6δ^{2}); 31.31 (6-NMe); 171.34 (7); 48.00 (7α); 14.92 (7β); 174.38 (8); 44.56 (8α); 17.66 (8β); 171.37 (9); 48.17 (9α); 39.24 (9β); 24.77 (9γ); 23.63 (9δ^{1}); 21.67 (9δ^{2}); 29.54 (9-NMe); 172.63 (10); 57.41 (10α); 40.59 (10β); 23.81 (10γ); 23.48 (10δ^{1}); 23.89 (10δ^{2}); 29.76 (10-NMe); 172.80 (11); 59.57 (11α); 29.30 (11β); 18.52 (11γ^{1}); 20.92 (11γ^{2}); 30.52 (11-NMe). HRMS, calcd for $C_{67}H_{121}N_{12}O_{13}$ $(M)^{+}$ 1301.9171, found 1301.9183; $[\alpha]_D^{20}$ −225 (*c* 1, $CHCl_3$).

5.3. [*O*-(4-Trimethylammonio-butanoyl)-MeBmt^{1}]-cyclosporin chloride (4)

Cyclosporin A (**1**; 1.2 g, 1 mmol) was dissolved in acetonitrile (3 mL) and treated with triethylamine (560 μL, 4 mmol), 4-dimethylaminobutyric acid (671 mg,

4 mmol), dicyclohexylcarbodiimide (DCCI; 830 mg, 4 mmol) and DMAP (4-dimethylamino pyridine; 100 mg). After stirring for 4 days at 50 °C the mixture was evaporated, the residue taken up in dichloromethane and filtered. After chromatography (gradient $CH_2Cl_2/CH_3OH/AcOH$ 15:1:0.1 to 9:1:0.2), 270 mg (20%) of 1-(4-dimethylamino-butanoyl)-MeBmt[1]-cyclosporin were obtained. The compound (270 mg, 0.21 mmol) was treated with methyl iodide (130 μL, 2.1 mmol) in acetone (16 mL) for 24 h at room temperature. After workup, the product was converted to the chloride as described under 5.2 to obtain 101 mg **4**; yield: 36%. TLC: $R_f = 0.24$ ($CH_2Cl_2/CH_3OH/H_2O$/AcOH 80:8:1:1); ^{1}H NMR ($CDCl_3$): 5.51 (1α); 5.50 (1β); 1.88 (1γ); 0.84 (1γ-Me); 2.07 + 1.55 (1δ); 5.11 (1ε); 5.28 (1ζ); 1.58 (1η); 3.19 (1-NMe); 2.48+2.40 ($1\beta^b$); 2.06 ($1\beta^c$); 3.65+3.47 ($1\beta^d$); 3.46 ($1\beta^d$-NMe_3); 4.90 (2α); 1.68 (2β); 0.84 (2γ); 8.54 (2-NH); 4.64+3.14 (3α); 3.42 (3-NMe); 5.35 (4α); 1.96+1.62 (4β); 1.43 (4γ); 0.93 ($4\delta^1$); 0.86 ($4\delta^2$); 3.06 (4-NMe); 4.75 (5α); 2.40 (5β); 0.99 ($5\gamma^1$); 0.79 ($5\gamma^2$); 7.47 (5-NH); 5.24 (6α); 2.23+1.07 (6β); 1.88 (6γ); 0.97 ($6\delta^1$); 0.73 ($6\delta^2$); 3.20 (6-NMe); 4.39 (7α); 1.28 (7β); 8.01 (7-NH); 4.82 (8α); 1.25 (8β); 7.49 (8-NH); 5.62 (9α); 2.18+1.14 (9β); 1.31 (9γ); 0.94 ($9\delta^1$); 0.83 ($9\delta^2$); 3.20 (9-NMe); 5.16 (10α); 1.84+1.32 (10β); 1.43 (10γ); 1.05 ($10\delta^1$); 1.02 ($10\delta^2$); 2.62 (10-NMe); 4.84 (11α); 2.13 (11β); 0.88 ($11\gamma^1$); 0.85 ($11\gamma^2$); 2.67 (11-NMe). ^{13}C NMR: 167.78 (1); 55.89 (1α); 74.45 (1β); 32.98 (1γ); 17.84 (1γ-Me); 33.69 (1δ); 128.56 (1ε); 126.89 (1ζ); 17.62 (1η); 32.27 (1-NMe); 171.93 ($1\beta^a$); 29.16 ($1\beta^b$); 18.22 ($1\beta^c$); 65.80 ($1\beta^d$); 53.27 ($1\beta^d$-NMe_3); 172.70 (2); 48.85 (2α); 24.99 (2β); 9.86 (2γ); 170.78 (3); 50.08 (3α); 39.21 (3-NMe); 169.84 (4); 55.25 (4α); 35.83 (4β); 24.77 (4γ); 23.73 ($4\delta^1$); 21.26 ($4\delta^2$); 31.29 (4-NMe); 173.52 (5); 54.70 (5α); 31.68 (5β); 19.54 ($5\gamma^1$); 18.18 ($5\gamma^2$); 171.31 (6); 54.32 (6α); 37.05 (6β); 24.53 (6γ); 24.00 ($6\delta^1$); 21.26 ($6\delta^2$); 31.29 (6-NMe); 171.27 (7); 48.19 (7α); 15.05 (7β); 174.01 (8); 44.69 (8α); 17.63 (8β); 171.06 (9); 48.09 (9α); 39.27 (9β); 24.78 (9γ); 22.96 ($9\delta^1$); 21.76 ($9\delta^2$); 29.64 (9-NMe); 171.00 (10); 57.13 (10α); 40.97 (10β); 24.26 (10γ); 23.97 ($10\delta^1$); 23.47 ($10\delta^2$); 29.86 (10-NMe); 172.84 (11); 58.68 (11α); 29.43 (11β); 18.60 ($11\gamma^1$); 20.56 ($11\gamma^2$); 30.30 (11-NMe). HRMS, calcd for $C_{69}H_{125}N_{12}O_{13}$ $(M)^+$ 1329.9484, found 1329.9479; $[\alpha]_D^{20}$ −224.9 (*c* 5, THF).

5.4. [2-[*O*-(11-Trimethylammonio-undecanoyl)]-ethyl-D-Ser[8]]-cyclosporin chloride (6)

A mixture of **5** (1 g, 0.8 mmol), 11-dimethylaminoundecanoic acid (310 mg, 2.37 mmol), dicyclohexyl carbodiimide (489 mg, 2.37 mmol) and 145 mg DMAP in CH_2Cl_2 (15 mL) was stirred at room temperature for 16 h. After filtration and evaporation, the residue was chromatographed ($CH_2Cl_2/CH_3OH/H_2O$/AcOH 90:10:1:1) to yield 188 mg (32%) of [2-[*O*-(11-dimethylammonio-undecanoyloxy)]-ethyl-D-Ser[8]]-cyclosporin. Quaternization of this intermediate (90 mg, 0.061 mmol) was done as described for **4** and, following conversion to the chloride as described under 5.2, afforded **6** in 80% yield. TLC: $R_f = 0.5$ ($CH_2Cl_2/CH_3OH/H_2O$/AcOH 80:8:1:1); ^{1}H NMR ($CDCl_3$; selected, diagnostically relevant signals): 5.49 (1α); 3.79 (1β); 0.71 (1γ-Me); 5.33 (1ε); 5.33 (1ζ); 3.50 (1-NMe); 5.00 (2α); 0.87 (2γ); 8.03 (2-NH); 4.72+3.22 (3α); 3.38 (3-NMe); 5.33 (4α); 3.09 (4-NMe); 4.65 (5α); 7.47 (5-NH); 5.01 (6α); 3.24 (6-NMe); 4.47 (7α); 1.36 (7β); 7.76 (7-NH); 5.10 (8α); 3.60 (8β); 7.00 (8-NH); 3.60 ($8\beta^a$); 4.14 ($8\beta^b$); 2.31 ($8\beta^d$); 3.50 ($8\beta^m$); 3.43 ($8\beta^m$-NMe_3); 5.72 (9α); 3.14 (9-NMe); 5.03 (10α); 2.68 (10-NMe); 5.11 (11α); 2.72 (11-NMe). ^{13}C NMR (selected, diagnostically relevant signals): 58.85 (1α); 74.5* (1β); 16.98 (1γ-Me); 129.3** (1ε); 126.3** (1ζ); 33.91 (1-NMe); 173.75 (2); 48.7* (2α); 9.83 (2γ); 171.0* (3); 50.2* (3α); 39.3* (3-NMe); 55.5* (4α); 31.1* (4-NMe); 173.5* (5); 55.4* (5α); 55.1* (6α); 31.4* (6-NMe); 171.4* (7); 48.9* (7α); 16.01 (7β); 171.4* (8); 48.4* (8α); 71.7* (8β); 69.3* ($8\beta^a$); 62.8* ($8\beta^b$); 173.5* ($8\beta^c$); 34.06 ($8\beta^d$); 67.32 ($8\beta^m$); 54.50 ($8\beta^m$-NMe_3); 170.3* (9); 48.58 (9α); 29.8* (9-NMe); 169.9* (10); 57.35 (10α); 29.7* (10-NMe); 173.4* (11); 57.94 (11α); 29.8* (11-NMe) [*/** value taken from HSQC/HMBC]. HRMS, calcd for $C_{78}H_{143}N_{12}O_{15}$ $(M)^+$ 1488.0790, found 1488.0789; $[\alpha]_D^{20}$ −83.2 (*c* 5, CH_3OH).

5.5. [2-[*O*-(2-Trimethylammonio-ethoxycarbonyl)]-ethyl-D-Ser[8]]-cyclosporin chloride (7)

5 (1.26 g, 1 mmol) and lutidine (460 μL, 4 mmol) were dissolved in dry CH_2Cl_2 (30 mL) and treated at 0 °C with phosgene (1.6 mL of a 1.93 M solution in toluene, 3 mmol). After stirring overnight at 0 °C, excess phosgene was removed at reduced pressure and 2-dimethylaminoethanol (202 μL, 2 mmol) was added. The solution was kept overnight at 0 °C, washed with water and evaporated. The residue was chromatographed (CH_2Cl_2/CH_3OH/AcOH = 9:1:0.1), freeze dried and passed through an LH-20 column (THF/water 7:3) to yield 134 mg (9.8%) of [2-[*O*-(2-dimethylamino-ethoxycarbonyl)]-ethyl-D-Ser[8]]-cyclosporin. The quarternization and subsequent conversion to the chloride was performed as described for **4**: from 220 mg (0.16 mmol) of the intermediate 175 mg (77%) of **7** were obtained. TLC: $R_f = 0.03$ ($CH_2Cl_2/CH_3OH/H_2O$/AcOH 80:8:1:1); ^{1}H NMR ($CDCl_3$): 5.45 (1α); 3.76 (1β); 1.69 (1γ); 0.67 (1γ-Me); 2.38+1.64 (1δ); 5.32 (1ε); 5.32 (1ζ); 1.60 (1η); 3.48 (1-NMe); 5.01 (2α); 1.70+1.59 (2β); 0.84 (2γ); 7.94 (2-NH); 4.72+3.19 (3α); 3.36 (3-NMe); 5.30 (4α); 1.98+1.59 (4β); 1.41 (4γ); 0.91 ($4\delta^1$); 0.84 ($4\delta^2$); 3.07 (4-NMe); 4.62 (5α); 2.38 (5β); 1.03 ($5\gamma^1$); 0.85 ($5\gamma^2$); 7.45 (5-NH); 4.96 (6α); 1.98+1.37 (6β); 1.57 (6γ); 0.92 ($6\delta^1$); 0.82 ($6\delta^2$); 3.21 (6-NMe); 4.41 (7α); 1.34 (7β); 7.77 (7-NH); 5.06 (8α); 3.58+3.43 (8β); 6.99 (8-NH); 3.62 ($8\beta^a$); 4.23 ($8\beta^b$); 4.65 ($8\beta^d$); 4.07 ($8\beta^e$); 3.43 ($8\beta^e$-NMe_3); 5.69 (9α); 2.05+1.19 (9β); 1.40 (9γ); 0.92 ($9\delta^1$); 0.85 ($9\delta^2$); 3.12 (9-NMe); 5.02 (10α); 2.08+1.22 (10β); 1.46 (10γ); 0.99 ($10\delta^1$); 0.98 ($10\delta^2$); 2.67 (10-NMe); 5.10 (11α); 2.12 (11β); 0.98 ($11\gamma^1$); 0.85 ($11\gamma^2$); 2.71 (11-NMe). ^{13}C NMR: 170.31 (1); 58.76 (1α); 74.71 (1β); 36.02 (1γ); 17.75 (1γ-Me); 35.59 (1δ); 129.61 (1ε); 126.24 (1ζ); 17.84 (1η); 33.95 (1-NMe); 173.78 (2); 48.81 (2α); 25.00 (2β); 9.83 (2γ); 171.11 (3); 50.31 (3α); 39.34 (3-NMe); 170.05 (4); 55.49 (4α); 35.98 (4β); 24.87 (4γ); 23.72 ($4\delta^1$); 21.90 ($4\delta^2$); 31.25 (4-NMe); 173.72 (5); 55.34 (5α); 31.08 (5β); 19.73 ($5\gamma^1$); 21.77 ($5\gamma^2$); 171.75 (6); 55.23 (6α); 37.37 (6β); 25.50 (6γ); 23.97

(6δ^1); 21.11 (6δ^2); 31.47 (6-NMe); 171.58 (7); 49.10 (7α); 16.15 (7β); 171.34 (8); 48.71 (8α); 71.73 (8β); 68.92 (8β^a); 67.32 (8β^b); 153.99 (8β^c); 61.62 (8β^d); 64.73 (8β^e); 54.48 (8β^e-NMe_3); 170.32 (9); 48.65 (9α); 39.11 (9β); 24.06 (9γ); 23.36 (9δ^1); 18.40 (9δ^2); 29.94 (9-NMe); 171.06 (10); 57.49 (10α); 40.59 (10β); 24.51 (10γ); 23.66 (10δ^1); 23.38 (10δ^2); 29.64 (10-NMe); 173.54 (11); 57.89 (11α); 29.06 (11β); 18.66 (11γ^1); 20.25 (11γ^2); 29.84 (11-NMe). HRMS, calcd for $C_{70}H_{127}N_{12}O_{16}$ $(M)^+$ 1391.9488, found 1391.9489; $[\alpha]_D^{20}$ −132.7 (*c* 5, THF).

5.6. [2-[*O*-(4-Trimethylammonio-butoxycarbamoyl)]-ethyl-D-Ser^8]-cyclosporin chloride (8)

5 (1.26 g, 1 mmol) and lutidine (460 μL, 4 mmol) were dissolved in dry CH_2Cl_2 (30 mL) and treated at 0 °C with phosgene (1.6 mL of a 1.93 M solution in toluene, 3 mmol). After stirring overnight at 0 °C, excess phosgene was removed at reduced pressure and 2-dimethylaminobutylamine (232 mg, 2 mmol) was added. The solution was stirred for 3 h at room temperature, washed with water, and evaporated. The residue was chromatographed (CH_2Cl_2/CH_3OH/AcOH 9:1:0.1) and the eluate was freeze dried. Yield: 222 mg (15%) of [2-[*O*-(4-dimethylamino-butoxycarbamoyl)]-ethyl-D-Ser^8]-cyclosporin. A solution of the compound (473 mg, 0.34 mmol) and CH_3I (1 mL) in acetone (20 mL) was kept for 2 h at room temperature and evaporated subsequently. The residue was first passed through a silica gel column (CH_2Cl_2/CH_3OH/AcOH/H_2O gradient 17:3:0.3:0 to 16:4:0.5:0.5), then over LH-20 (THF/water 7:3) and finally through an Amberlyst IRA 401S (chloride form) column. Yield 220 mg (44%). TLC: R_f = 0.56 (CH_2Cl_2/CH_3OH/AcOH/H_2O 80:25:5:5); ^{1}H NMR ($CDCl_3$): 5.44 (1α); 3.76 (1β); 1.60 (1γ); 0.68 (1γ-Me); 2.37+1.67 (1δ); 5.32 (1ε); 5.32 (1ζ); 1.60 (1η); 3.48 (1-NMe); 4.99 (2α); 1.70+1.58 (2β); 0.85 (2γ); 7.91 (2-NH); 4.72+3.19 (3α); 3.36 (3-NMe); 5.30 (4α); 1.96+1.60 (4β); 1.40 (4γ); 0.98 (4δ^1); 0.84 (4δ^2); 3.06 (4-NMe); 4.63 (5α); 2.38 (5β); 1.03 (5γ^1); 0.85 (5γ^2); 7.44 (5-NH); 4.97 (6α); 1.96+1.38 (6β); 1.58 (6γ); 0.90 (6δ^1); 0.84 (6δ^2); 3.21 (6-NMe); 4.41 (7α); 1.33 (7β); 7.78 (7-NH); 5.05 (8α); 3.57+3.46 (8β); 7.00 (8-NH); 3.63+3.56 (8β^a); 4.09 (8β^b); 6.29 (8β^d); 3.20 (8β^e); 1.63 (8β^f); 1.85 (8β^g); 3.58 (8β^h); 3.32 (8β^h-NMe_3); 5.68 (9α); 2.07+1.21 (9β); 1.42 (9γ); 0.92 (9δ^1); 0.87 (9δ^2); 3.13 (9-NMe); 5.04 (10α); 2.09+1.20 (10β); 1.45 (10γ); 0.98 (10δ^1); 0.97 (10^2); 2.66 (10-NMe); 5.11 (11α); 2.11 (11β); 0.98 (11γ^1); 0.85 (11γ^2); 2.71 (11-NMe). ^{13}C NMR: 170.40 (1); 58.69 (1α); 74.71 (1β); 36.03 (1γ); 17.71 (1γ-Me); 35.61 (1δ); 129.59 (1ε); 126.26 (1ζ); 17.84 (1η); 33.94 (1-NMe); 173.75 (2); 48.83 (2α); 25.03 (2β); 9.83 (2γ); 171.05 (3); 50.32 (3α); 39.30 (3-NMe); 170.05 (4); 55.48 (4α); 35.98 (4β); 24.88 (4γ); 23.75 (4δ^1); 21.90 (4δ^2); 31.23 (4-NMe); 173.68 (5); 55.36 (5α); 31.07 (5β); 19.71 (5γ^1); 21.82 (5γ^2); 171.76 (6); 55.26 (6α); 37.39 (6β); 25.49 (6γ); 23.75 (6δ^1); 21.13 (6δ^2); 31.49 (6-NMe); 171.53 (7); 49.08 (7α); 16.22 (7β); 171.37 (8); 48.83 (8α); 71.61 (8β); 69.80 (8β^a); 63.43 (8β^b); 153.87 (8β^c); 39.50 (8β^e); 26.47 (8β^f); 20.04 (8β^g); 66.36 (8β^h); 54.48 (8β^h-NMe_3); 170.31 (9); 48.65 (9α); 39.14 (9β); 24.07 (9γ); 23.35 (9δ^1); 18.46 (9δ^2); 30.03 (9-NMe); 171.02 (10); 57.48 (10α); 40.65 (10β); 24.56 (10γ); 23.69 (10δ^1); 23.19 (10δ^2); 29.77 (10-NMe); 173.51 (11); 57.89 (11α); 28.99 (11β); 18.68 (11γ^1); 20.22 (11γ^2); 29.84 (11-NMe). HRMS, calcd for $C_{72}H_{132}N_{13}O_{15}$ $(M)^+$ 1418.9960, found 1418.9953; $[\alpha]_D^{20}$ −162.0 (*c* 5, THF).

5.7. [2-[*O*-(Succinic acid mono-(2-oxyethyl) ester)]-D-Ser^8]-cyclosporin (9)

A solution of **5** (200 mg, 0.16 mmol), succinic anhydride (20 mg, 0.2 mmol) and DMAP (10 mg) in acetone (30 mL) was stirred for 24 h at room temperature. After evaporation, the residue was chromatographed (CH_2Cl_2/CH_3OH 7:1) affording 114 mg (52.8%) of a colorless foam. TLC: R_f = 0.8 (CH_2Cl_2/CH_3OH 7:1); ^{1}H NMR ($CDCl_3$): 5.48 (1α); 3.74 (1β); 1.60 (1γ); 0.67 (1γ-Me); 2.39+1.63 (1δ); 5.32 (1ε); 5.32 (1ζ); 1.61 (1η); 3.49 (1-NMe); 5.01 (2α); 1.69+1.58 (2β); 0.84 (2γ); 7.98 (2-NH); 4.72+3.19 (3α); 3.37 (3-NMe); 5.32 (4α); 1.98+1.60 (4β); 1.41 (4γ); 0.92 (4δ^1); 0.85 (4δ^2); 3.08 (4-NMe); 4.63 (5α); 2.40 (5β); 1.04 (5γ^1); 0.82 (5γ^2); 7.46 (5-NH); 4.95 (6α); 2.01+1.38 (6β); 1.67 (6γ); 0.93 (6δ^1); 0.84 (6δ^2); 3.22 (6-NMe); 4.49 (7α); 1.35 (7β); 7.75 (7-NH); 5.11 (8α); 3.61+3.42 (8β); 7.00 (8-NH); 3.62+3.57 (8β^a); 4.23+4.15 (8β^b); 2.62 (8β^d); 2.62 (8β^e); 5.70 (9α); 2.12+1.18 (9β); 1.43 (9γ); 0.92 (9δ^1); 0.86 (9δ^2); 3.12 (9-NMe); 5.03 (10α); 2.04+1.28 (10β); 1.46 (10γ); 1.02 (10δ^1); 0.99 (10δ^2); 2.67 (10-NMe); 5.10 (11α); 2.12 (11β); 1.00 (11γ^1); 0.85 (11γ^2); 2.72 (11-NMe). ^{13}C NMR: 170.31 (1); 58.78 (1α); 74.85 (1β); 36.09 (1γ); 16.80 (1γ-Me); 35.66 (1δ); 129.65 (1ε); 126.21 (1ζ); 17.83 (1η); 34.07 (1-NMe); 173.90 (2); 48.78 (2α); 24.95 (2β); 9.84 (2γ); 171.21 (3); 50.32 (3α); 39.42 (3-NMe); 170.05 (4); 55.50 (4α); 36.00 (4β); 24.88 (4γ); 23.70 (4δ^1); 21.93 (4δ^2); 31.27 (4-NMe); 173.84 (5); 55.34 (5α); 31.11 (5β); 19.78 (5γ^1); 21.69 (5γ^2); 172.05 (6); 55.40 (6α); 37.39 (6β); 25.51 (6γ); 23.78 (6δ^1); 21.12 (6δ^2); 31.51 (6-NMe); 171.63 (7); 49.11 (7α); 16.14 (7β); 171.65 (8); 48.98 (8α); 71.82 (8β); 69.31 (8β^a); 63.91 (8β^b); 171.69 (8β^c); 29.57 (8β^d); 28.94 (8β^e); 173.75 (8β^f); 170.37 (9); 48.74 (9α); 39.08 (9β); 23.94 (9γ); 23.41 (9δ^1); 18.37 (9δ^2); 29.96 (9-NMe); 170.22 (10); 57.51 (10α); 40.49 (10β); 24.43 (10γ); 23.67 (10δ^1); 23.36 (10δ^2); 29.77 (10-NMe); 173.69 (11); 57.89 (11α); 29.19 (11β); 18.65 (11γ^1); 20.36 (11γ^2); 29.94 (11-NMe). HRMS, calcd for $C_{68}H_{118}N_{11}O_{17}Na$ $(M+Na)^+$ 1384.8677, found 1384.8674; $[\alpha]_D^{20}$ −150.0 (*c* 5, CH_3OH).

5.8. [2-[*O*-(Phosphate)]-ethyl-D-Ser^8]-cyclosporin (10)

A mixture of **5** (1.26 g, 1 mmol) and lutidine (950 μL, 8 mmol) in trimethyl phosphate (6 mL) was treated at 0 °C with phosphoryl chloride (910 μL, 10 mmol) for 3 h and then poured into an aqueous bicarbonate solution. The mixture was acidified with 1 N HCl (pH 1), extracted with CH_2Cl_2 and chromatographed (CH_2Cl_2/CH_3OH/AcOH 9:1:0.1). Yield: 935 mg (69.6%). TLC: R_f = 0.51 (CH_2Cl_2/CH_3OH/H_2O/AcOH 80:25:5:5); ^{1}H NMR ($CDCl_3$): 5.42 (1α); 3.77 (1β); 1.60 (1γ); 0.69 (1γ-Me); 2.37+1.61 (1δ); 5.34 (1ε); 5.34 (1ζ); 1.61 (1η); 3.49 (1-NMe); 4.99 (2α); 1.71+1.59 (2β); 0.85 (2γ); 7.91 (2-NH); 4.72+3.19 (3α); 3.35 (3-NMe); 5.30 (4α);

1.96+1.60 (4β); 1.42 (4γ); 0.95 (4δ^1); 0.85 (4δ^2); 3.06 (4-NMe); 4.64 (5α); 2.38 (5β); 1.03 (5γ^1); 0.82 (5γ^2); 7.41 (5-NH); 5.05 (6α); 1.98+1.41 (6β); 1.67 (6γ); 0.99 (6δ^1); 0.86 (6δ^2); 3.20 (6-NMe); 4.51 (7α); 1.33 (7β); 7.92 (7-NH); 5.05 (8α); 3.64+3.52 (8β); 7.30 (8-NH); 3.64+3.61 (8β^a); 4.07 (8β^b); 5.69 (9α); 2.16+1.24 (9β); 1.43 (9γ); 0.93 (9δ^1); 0.87 (9δ^2); 3.18 (9-NMe); 5.05 (10α); 2.10+1.22 (10β); 1.45 (10γ); 1.01 (10δ^1); 0.99 (10δ^2); 2.68 (10-NMe); 5.13 (11α); 2.13 (11β); 0.98 (11γ^1); 0.84 (11γ^2); 2.73 (11-NMe). ^{13}C NMR: 170.38 (1); 58.55 (1α); 74.78 (1β); 36.8* (1γ); 16.89 (1γ-Me); 35.61 (1δ); 129.57 (1ϵ); 126.27 (1ζ); 17.85 (1η); 33.94 (1-NMe); 173.80 (2); 48.93 (2α); 25.03 (2β); 9.85 (2γ); 171.00 (3); 50.32 (3α); 39.29 (3-NMe); 170.04 (4); 55.52 (4α); 36.0* (4β); 24.89 (4γ); 23.65 (4δ^1); 21.86 (4δ^2); 31.19 (4-NMe); 173.66 (5); 55.35 (5α); 31.10 (5β); 19.69 (5γ^1); 21.68 (5γ^2); 171.86 (6); 55.42 (6α); 37.29 (6β); 25.42 (6γ); 23.77 (6δ^1); 21.19 (6δ^2); 31.47 (6-NMe); 171.58 (7); 48.86 (7α); 16.25 (7β); 171.42 (8); 49.13 (8α); 71.23 (8β); 70.45 (8β^a); 65.7* (8β^b); 170.49 (9); 48.77 (9α); 39.14 (9β); 24.21 (9γ); 23.34 (9δ^1); 18.48 (9δ^2); 30.13 (9-NMe); 170.04 (10); 57.46 (10α); 40.61 (10β); 24.54 (10γ); 23.65 (10δ^1); 23.04 (10δ^2); 29.81 (10-NMe); 173.58 (11); 57.89 (11α); 29.03 (11β); 18.69 (11γ^1); 20.25 (11γ^2); 29.94 (11-NMe) [* value taken from HSQC]. ^{31}P-NMR ($CDCl_3$): 2.3 (8β^c). HRMS, calcd for $C_{64}H_{115}N_{11}O_{17}$ PNa $(M+Na)^+$ 1364.8181, found 1364.8186; $[\alpha]_D^{20}$ −187.2 (*c* 10, acetone).

5.9. [2-[*O*-(Phosphate monoethylester)]-ethyl-D-Ser^8]-cyclosporin (11)

A mixture of **5** (243 mg, 0.19 mmol) and lutidine (80 μL, 0.665 mmol) in trimethyl phosphate (0.5 mL) was treated at 0 °C with phosphoryl chloride (910 μL, 1.0 mmol) for 1 h. Then, ethanol (30.7 μL, 0.34 mmol) was added. After stirring overnight at room temperature, the solution was poured in an aqueous bicarbonate solution, stirred, acidified with 1 N HCl (pH 1), extracted with CH_2Cl_2 and evaporated. The residue was first chromatographed over silica gel (CH_2Cl_2/CH_3OH/AcOH 9:1:0.1) and subsequently passed through a Dowex 50 × 8 Na^+ column (THF/water 7:3). Yield (sodium salt): 79 mg (29.9%). TLC: R_f = 0.22 (CH_2Cl_2/CH_3OH/H_2O/AcOH 80:8:1:1); HRMS, calcd for $C_{66}H_{119}N_{11}O_{17}$PNa $(M+Na)^+$ 1392.8494, found 1392.8489; $[\alpha]_D^{20}$ −127.0 (*c* 5, acetone).

5.10. [2-[*O*-(Phosphocholine ester)]-ethyl-D-Ser^8]-cyclosporin (12)

To a solution of **5** (631 mg, 0.5 mmol) and lutidine (480 μL, 4 mmol) in trimethyl phosphate (2.5 mL) 2-bromoethyl phosphorodichloridate[15] (1.03 g, 5 mmol) was added dropwise at 0 °C. After stirring at room temperature for 4 h, the mixture was poured onto ice water and extracted with CH_2Cl_2. Chromatography (CH_2Cl_2/CH_3OH/AcOH 9:1:0.2) afforded 465 mg (64%) of [2-[*O*-(phosphate 2-bromo ethylester)]-ethyl-D-Ser^8]-cyclosporin (yellowish oil). The product was not stable and was used immediately in the next step. A mixture of 150 mg (0.1 mmol) of the intermediate, trimethylamine (0.5 mL), $CHCl_3$ (0.3 mL), and isopropanol (0.5 mL) was kept at 50 °C in an autoclav. After evaporation in vacuo and chromatography (gradient CH_2Cl_2/CH_3OH/H_2O 8:2:0.1 to 15:4:1) 111 mg (78%) of **12** were obtained as colorless foam. TLC: R_f = 0.53 (CH_2Cl_2/CH_3OH/H_2O/AcOH 80:25:5:5); ^{1}H NMR ($CDCl_3$): 5.48 (1α); 3.76 (1β); 1.60 (1γ); 0.68 (1γ-Me); 2.38+1.62 (1δ); 5.34 (1ε); 5.34 (1ζ); 1.62 (1η); 3.50 (1-NMe); 5.02 (2α); 1.72+1.60 (2β); 0.85 (2γ); 7.92 (2-NH); 4.73+3.20 (3α); 3.37 (3-NMe); 5.32 (4α); 1.96+1.63 (4β); 1.42 (4γ); 0.95 (4δ^1); 0.83 (4δ^2); 3.09 (4-NMe); 4.64 (5α); 2.40 (5β); 1.05 (5γ^1); 0.86 (5γ^2); 7.46 (5-NH); 5.95 (6α); 1.99 + 1.41 (6β); 1.61 (6γ); 0.96 (6δ^1); 0.87 (6δ^2); 3.22 (6-NMe); 4.39 (7α); 1.35 (7β); 7.74 (7-NH); 5.02 (8α); 3.62 + 3.43 (8β); 7.00 (8-NH); 3.98+3.93 (8β^a); 4.02 (8β^b); 4.34 (8β^d); 3.78 (8β^e); 3.34 (8β^e-NMe_3); 5.71 (9α); 2.10+1.22 (9β); 1.43 (9γ); 0.93 (9δ^1); 0.87 (9δ^2); 3.15 (9-NMe); 5.05 (10α); 2.09 + 1.24 (10β); 1.48 (10γ); 1.00 (10δ^1); 1.02 (10δ^2); 2.68 (10-NMe); 5.13 (11α); 2.14 (11β); 1.01 (11γ^1); 0.86 (11γ^2); 2.73 (11-NMe). ^{13}C NMR: 170.32 (1); 58.89 (1α); 74.87 (1β); 36.19 (1γ); 16.75 (1γ-Me); 35.75 (1δ); 129.62 (1ϵ); 126.27 (1ζ); 17.86 (1η); 33.84 (1-NMe); 173.83 (2); 49.07 (2α); 24.98 (2β); 9.85 (2γ); 171.17 (3); 50.31 (3α); 39.41 (3-NMe); 170.02 (4); 55.52 (4α); 36.01 (4β); 24.90 (4γ); 23.74 (4δ^1); 21.90 (4δ^2); 31.28 (4-NMe); 173.79 (5); 55.37 (5α); 31.08 (5β); 19.79 (5γ^1); 21.82 (5γ^2); 171.79 (6); 55.41 (6α); 37.51 (6β); 25.57 (6γ); 23.85 (6δ^1); 21.13 (6δ^2); 31.54 (6-NMe); 171.57 (7); 49.15 (7α); 16.32 (7β); 171.50 (8); 48.80 (8α); 71.28 (8β); 64.35 (8β^a); 63.0* (8β^b); 59.22 (8β^d); 66.62 (8β^e); 54.62 (8β^e-NMe_3); 170.39 (9); 48.67 (9α); 39.10 (9β); 24.07 (9γ); 23.37 (9δ^1); 18.43 (9δ^2); 30.03 (9-NMe); 170.10 (10); 57.54 (10α); 40.61 (10β); 24.52 (10γ); 23.74 (10δ^1); 23.31 (10δ^2); 29.76 (10-NMe); 173.58 (11); 57.86 (11α); 29.05 (11β); 18.70 (11γ^1); 20.31 (11γ^2); 29.87 (11-NMe) [* value taken from HSQC]. ^{31}P-NMR ($CDCl_3$): 0.5 (8β^c). HRMS, calcd for $C_{69}H_{128}N_{12}O_{17}P$ $(M)^+$ 1427.9253, found 1427.9246; $[\alpha]_D^{20}$ −189.0 (*c* 5, CH_3OH).

5.11. [2-[*O*-Sulfate]-ethyl-D-Ser^8]-cyclosporin (13)

NaH (0.5 g of a 55% suspension in dry THF, 11.45 mmol) was placed in a reaction flask under an inert atmosphere (Argon). A solution of D-Ser^8-cyclosporin (6.5 g, 5.34 mmol) in dry THF (75 mL) was added quickly to the NaH. After 1 h at room temperature a solution of glycol sulfate (Bachem) (1.0 g, 8.06 mmol) in THF (25 mL) was added over a period of 5 min. The temperature of the reaction mixture rose to 30 °C. After stirring for 2 h, H_2O (100 mL) was added dropwise. The mixture was diluted with 500 mL MTBE (methyl *tert*-butyl ether) and extracted with water (2 × 100 mL). The combined aqueous phases were saturated with NaCl and extracted with ethyl acetate (3 × 300 mL). The organic phases were dried over Na_2SO_4, filtered, and evaporated. 6.39 g of pure **13** were obtained (85%). ^{1}H NMR ($CDCl_3$): 5.42 (1α); 3.78 (1β); 1.62 (1γ); 0.69 (1γ-Me); 2.41+1.68 (1δ); 5.36 (1ε); 5.35 (1ζ); 1.62 (1η); 3.48 (1-NMe); 5.00 (2α); 1.72+1.60 (2β); 0.85 (2γ); 7.73 (2-NH); 4.74+3.24 (3α); 3.35 (3-NMe); 5.30 (4α); 1.96+1.61 (4β); 1.43 (4γ); 1.01 (4δ^1); 0.87 (4δ^2); 3.08 (4-NMe); 4.64 (5α); 2.36 (5β); 1.04

($5\gamma^1$); 0.86 ($5\gamma^2$); 7.40 (5-NH); 4.97 (6α); 1.96+1.45 (6β); 1.63 (6γ); 0.91 ($6\delta^1$); 0.87 ($6\delta^2$); 3.20 (6-NMe); 4.45 (7); 1.35 (7β); 7.71 (7-NH); 5.09 (8α); 3.58 (8β); 3.70+3.63 ($8\beta^1$); 4.13 ($8\beta^2$); 7.10 (8-NH); 5.66 (9α); 2.09+1.23 (9β); 1.40 (9γ); 0.93 ($9\delta^1$); 0.88 ($9\delta^2$); 3.16 (9-NMe); 5.04 (10α); 2.14 + 1.19 (10β); 1.48 (10γ); 0.95 ($10\delta^1$); 0.99 ($10\delta^2$); 2.69 (10-NMe); 5.15 (11α); 2.14 (11β); 0.99 ($11\gamma^1$); 0.85 ($11\gamma^2$); 2.76 (11-NMe). ^{13}C NMR: 170.20 (1); 58.77 (1α); 74.83 (1β); 36.30 (1γ); 16.54 (1γ-Me); 35.80 (1δ); 129.55 (1ϵ); 126.31 (1ζ); 17.89 (1η); 34.08 (1-NMe); 173.88 (2); 48.88 (2α); 25.06 (2β); 9.86 (2γ); 170.85 (3); 50.39 (3α); 39.25 (3-NMe); 170.01 (4); 55.42 (4α); 36.01 (4β); 24.91 (4γ); 23.78 ($4\delta^1$); 22.01 ($4\delta^2$); 31.01 (4-NMe); 173.72 (5); 55.42 (5α); 31.01 (5β); 19.67 ($5\gamma^1$); 21.86 ($5\gamma^2$); 172.31 (6); 55.42 (6α); 37.23 (6β); 25.57 (6γ); 23.67 ($6\delta^1$); 21.15 ($6\delta^2$); 31.56 (6-NMe); 171.68 (7); 49.34 (7α); 16.54 (7β); 171.85 (8); 49.65 (8α); 71.47 (8β); 69.83 ($8\beta^1$); 66.97 ($8\beta^2$); 170.40 (9); 48.97 (9α); 38.95 (9β); 24.27 (9γ); 23.35 ($9\delta^1$); 18.52 ($9\delta^2$); 30.11 (9-NMe); 169.93 (10); 57.43 (10α); 40.76 (10β); 24.64 (10γ); 23.64 ($10\delta^1$); 23.14 ($10\delta^2$); 29.74 (10-NMe); 173.52 (11); 57.87 (11α); 28.82 (11β); 18.76 ($11\gamma^1$); 20.10 ($11\gamma^2$); 29.86 (11-NMe). HRMS, calcd for $C_{69}H_{125}N_{12}O_{13}$ $(M-H+2Na)^+$ 1386.7904, found 1386.7893; $[\alpha]_D^{20}$ −173 (*c* 1.0, $CHCl_3$).

5.12. Biology

5.12.1. Skin donors and skin preparation. Rat skin was obtained from 8–10 week old hairless female animals (strain ICO:OFA hr-hr), purchased from Iffa-Credo (Lyon, France). After suffocation with CO_2 the skin was dissected and subcutaneous tissue was removed.

Human breast skin was obtained from cosmetic surgery, and was used within 16 h following excision. The skin was dermatomed to 0.4 mm with an Aesculap dermatome (Tuttlingen, Germany).

5.12.2. Penetration assay. Percutaneous penetration was studied in vitro using static Franz-type diffusion cells.[18] With the cells used here, the exposed skin area was 2.54 cm^2; the volume of the receptor chamber was 5.8 mL. Phosphate buffered saline/ethanol 3:1 was used as receptor phase, maintaining sink conditions throughout the experiment (maximum receptor concentrations of the drugs were at least 30-fold above their solubility in the receptor). All experiments were performed at 32 °C in triplicates for 48 h. An infinite dosing regimen was used: the test compounds were applied to the epicutaneous side of the skin in propylene glycol/oleyl alcohol at a concentration of 1% (w/v) in a volume of 300 μL.

Samples of 100 μL were withdrawn from the receptor phase at 8 time points during the 48-h experiment and replaced by fresh receptor fluid. After addition of an internal standard, these samples were analyzed by reversed-phase HPLC with UV detection (215 nm).

At the end of the experiment at 48 h, the skin was taken from the diffusion cells and the stratum corneum was removed by 5 strippings (rat skin) or 20 strippings (human skin) with transparent adhesive tape (Kores, Spain). Specimens from the stripped skin were taken with a biopsy punch, weighed and then homogenized in 0.2 M phosphate buffer (pH 6). Following addition of an internal standard, the homogenates were extracted with ethyl acetate/methanol (9:1 v/v). The extracts were purified using solid phase extraction (Supelclean LC-CN columns, Sigma–Aldrich) and then analyzed by reversed-phase HPLC. Extraction yields were in the range of 78–93%, depending on the compound but without significant differences between rat and human skin.

Drug concentrations in the skin and in the receptor fluid were calculated by comparing the area of drug versus the area of internal standard. Calibration curves were prepared for the test compound in the respective matrix (rat or human skin and receptor), and analyzed by linear regression. Permeation rates were calculated from receptor fluid concentrations by linear regression.[11]

All skin concentrations and permeation rates given are means of three experiments. Standard deviation was less than 20% in all cases.

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