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Synthesis of Rapamycin-Peptide Hybrid Molecule (RAP-P): High Affinity FKBP12 Ligand

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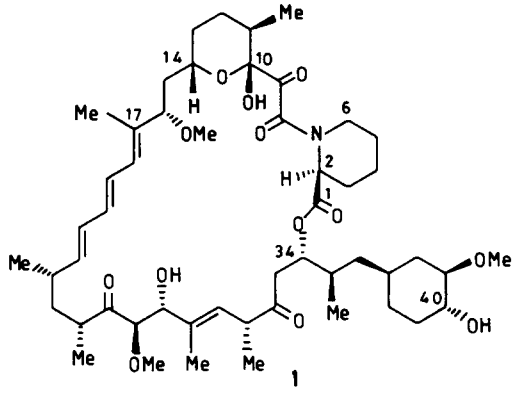
Abstract : The syntheses of two rapamycin-peptide hybrid molecules RAP-P (5 and 6) containing the rapamycin-FK 506-ascomycin binding domain and different peptide tethers are described in detail.

The macrocyclic immunosuppressants rapamycin (**1**)[1], ascomycin (**2**)[2], FK506 (**3**)[3], and cyclosporin (**4**)[4] are of dual-domain nature. All of them bind to the immunophilins, a class of ubiquitous cytosolic proteins, and the composite surface of the immunosuppressant-immunophilin complex, then, binds to other cellular proteins involved in signal transduction[5-8]. The first cellular target of cyclosporin is cyclophilin[9], while ascomycin, FK506 and rapamycin all bind to FKBP12[10,11]. The cyclophilin-cyclosporin, FKBP12-ascomycin and FKBP12-FK506 complexes bind to a second receptor protein, calcineurin[12], whereas the direct target of FKBP12-rapamycin is FRAP[13], also called TOR[14] or RAFT1[15].

These small organic molecules, thus, act as 'molecular adapters' to bridge, noncovalently, two distinct proteins together for biological action. This dual-domain model for the mechanism of action of these compounds poses significant challenge to structure-based design of analogous ligands. Such designed analogs will, not only, have to bind strongly to immunophilins, the complexes of these ligands and immunophilins will, in turn, have to effectively block the function of the second receptor protein. The first step towards designing an effective analog is, therefore, to achieve an excellent binding with immunophilin.

Recently, we have designed, synthesized and biologically evaluated a hybrid molecule, RAP-P (**5**)[16a], containing the rapamycin-FK506-ascomycin binding domain and a peptide tether, as part of our ongoing project on the development of effective analogs of macrocyclic immunosuppressants. These natural products are possibly mimicking some endogenous peptidal substances which made us interested in designing and synthesizing cyclic peptide-based analogs of these molecules[16b,c].

The conformation of rapamycin's binding domain as present in its FKBP12-bound form and determined by X-ray crystallographic analysis of the FKBP12-rapamycin complex[17] was chosen as the most desired one for optimum binding. Detailed computer modeling was then carried out[16] using tri-, tetra- and pentapeptide



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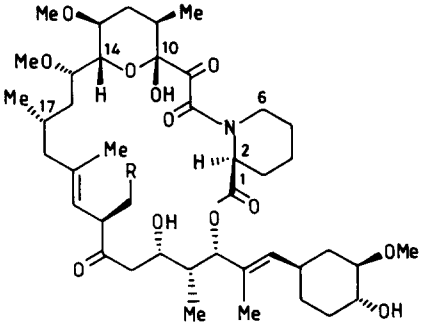
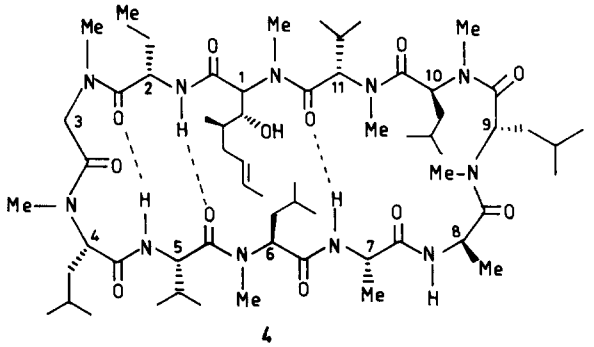


2 (R = Me)

3 (R = CH=CH$_2$)



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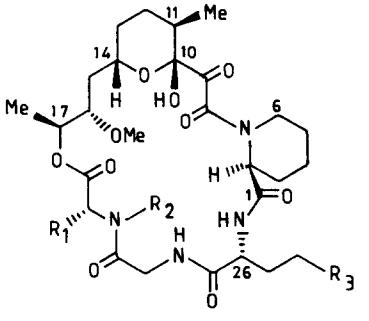


5 (R_1 = H, R_2 = Me, R_3 = C_6H_5)

6 (R_1 = Et, R_2 = H, R_3 = C_6H_{11})

cassettes to determine the most suitable tether to constrain the binding domain of the designed analog in this preferred conformation. These molecular modeling studies led us to conclude that the 21-membered designed ring system **5**, which is of the same size as FK506 and ascomycin and is having D-homoPhe-Gly-Sar as the peptide cassette, was the best candidate in term of its rigidity and ability to 'lock' the rapamycin/FK506/ascomycin binding domain in the desired conformation for strong binding with FKBP12. The D-isomer of homoPhe was chosen to provide the best fit for the C-26 stereocenter of FK506/ascomycin (or the C-34 center of rapamycin).

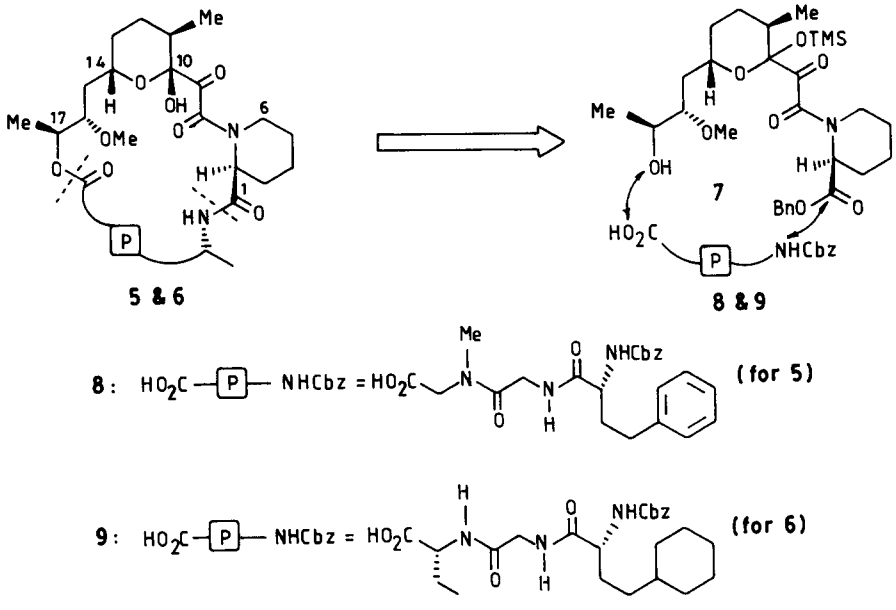
In this paper, we describe in detail the total synthesis of **5** and another member of this series **6** with a different peptide tether, D-γ-cHexAbu-Gly-D-Abu. Superimposition of the energy minimized structure of **5/6** and the FKBP12-bound FK506[18] showed the proximity between C-20 center of **5/6** and C-21 of FK506/ascomycin. Thats why it was decided to make an analog with an ethyl group on C-20 and not on N-21 which carried a methyl group in **5**. This, it was hoped, might lead to an improved effector domain. Same reasoning was behind the choice of γ-cyclohexyl-α-aminobutyric acid (γ-cHexAbu) to substitute the cyclohexyl appendage of FK506/ascomycin/rapamycin. The modular nature of these designed molecules should make

it possible to generate a series of compounds with different effector domains for targetting either calcineurin or FRAP (TOR/RAFT1) or both, as potential biological tools and immunosuppressive agents.

Results and Discussion

The basic strategy followed for the synthesis of such hybrid molecules is depicted in Scheme 1. The C-17 hydroxyl of the binding domain segment 7 was coupled with the carboxyl group of the peptide tethers, **8** (or **9**). This was then followed by the crucial macrolactamization step. This allows the synthesis of any such hybrid molecule having the rapamycin/FK506/ascomycin binding domain and any desired peptide tether.

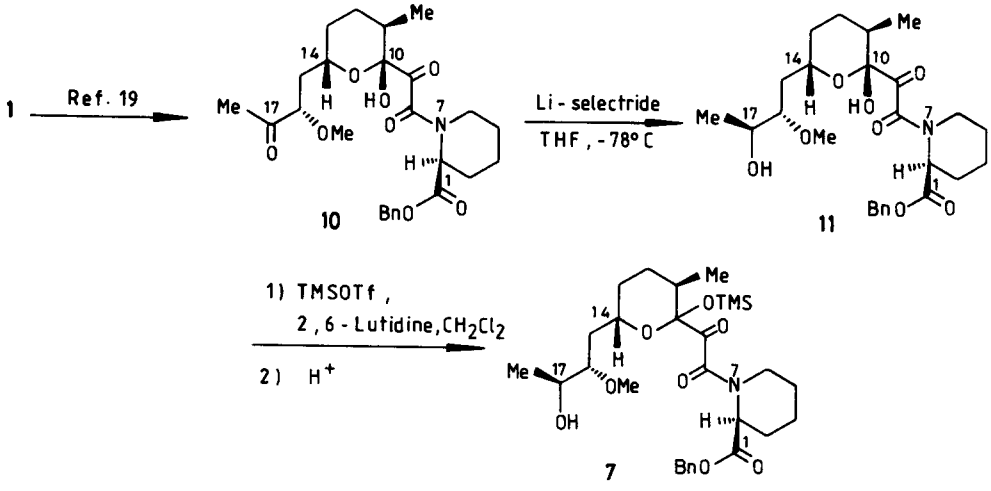
Scheme 1



The synthesis started with compound **10** (Scheme 2), itself prepared in two steps[19] from rapamycin. Diastereo- and chemoselective reduction of compound **10** with lithium tri-s-butylborohydride (Li-selectride) at -78°C in THF gave a single diastereomer **11** in 96% yield. But, the 'TLC-single-spot' compound was actually a mixture of rotamers in chloroform as well as in dimethylsulfoxide (ca 1:1) at room temperature as indicated by its ^{1}H NMR spectrum. It was stable at higher temperature and ^{1}H NMR in DMSO-d_6 at 150°C showed a single conformer. The newly generated methyl carbinol center was assigned the S-configuration based on the non-chelation controlled Anh-Eisenstein model[20]. Disilylation of compound **11** with trimethylsilyl trifluoromethane sulfonate (TMSOTf) followed by brief exposure to dilute acid to deprotect the C-17 hydroxyl gave the hemiketal-protected derivative 7 in 92% yield (~1:1 mixture of diastereomers)[21]. Once again it was

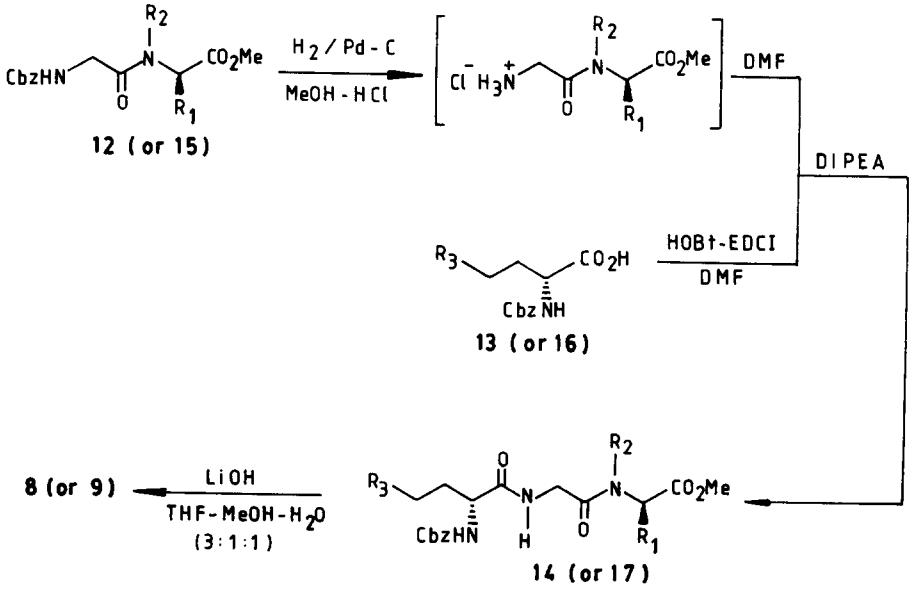
a mixture of rotamers in chloroform and dimethyl sulfoxide. It was also stable at higher temperature and ^1H NMR in DMSO-d_6 at 150°C showed just two diastereomers (ca 1:1).

Scheme 2



The peptide cassettes were synthesized following routine chemistry (Scheme 3). Cbz-Gly-Sar-OMe (**12**) was subjected to hydrogenation using Pd-C as catalyst in methanol containing 1.1 molar equivalent of

Scheme 3

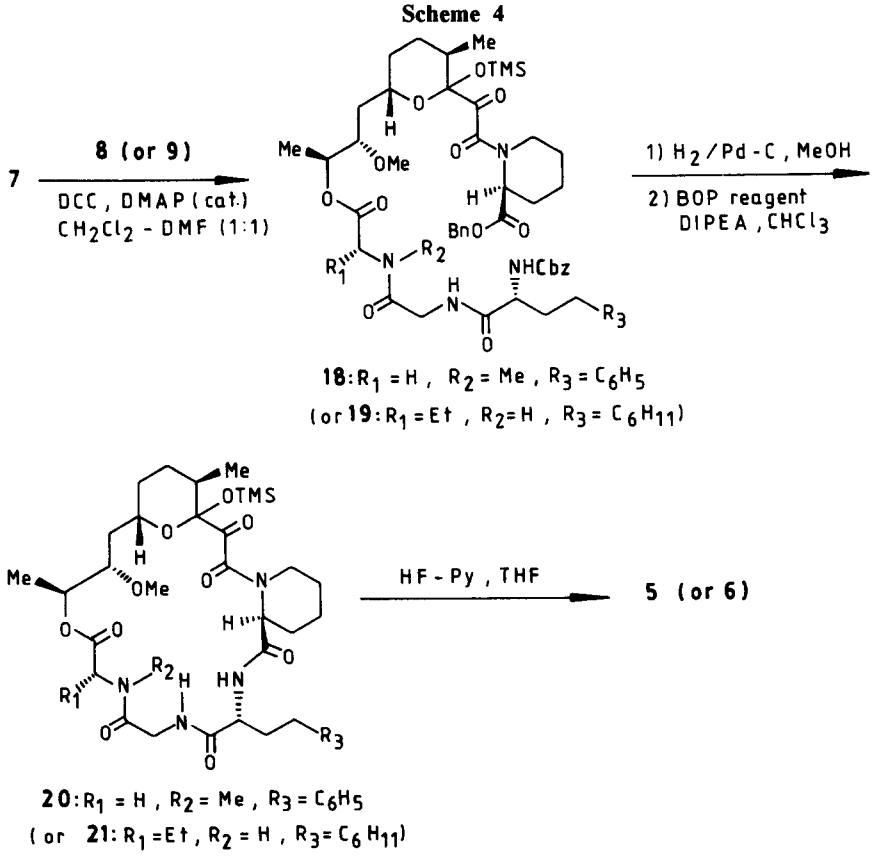


HCl (1N HCl, aqueous). The resulting amine hydrochloride, neutralized with N,N-diisopropylethylamine (DIPEA) was coupled with Cbz-D-homoPhe-OH (**13**) using 1-hydroxy benzotriazole (HOBt) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) in N,N-dimethylformamide (DMF) to get Cbz-D-homo-Phe-Gly-Sar-OMe (**14**). Saponification of **14** with LiOH in THF:MeOH:H_2O (3:1:1) at 0°C gave the requisite acid **8** to be coupled with **7**.

The same protocol was followed to synthesize the peptide cassette for **6**. The D-homoPhe-OH was reduced by hydrogenation using Pt black catalyst at 50 psi for 16 h to get D-γ-cyclohexyl-α-aminobutyric acid (D-γ-cHexAbu-OH)[22] which was Cbz-protected. The coupling of H-Gly-D-Abu-OMe, prepared by catalytic hydrogenation of Cbz-Gly-D-Abu-OMe (**15**) in presence of acid, with Cbz-D-γ-cHexAbu-OH (**16**) was done following the same procedure as described above. The tripeptide **17**, on saponification, gave the desired acid **9**.

The final stages of the synthesis, i.e., anchoring of these peptide cassettes to the rapamycin/FK506/ascomycin binding domain 7 is described in Scheme 4. The esterification step, i.e., coupling of the C-terminus of the peptide tether with C-17 hydroxyl was achieved using N,N'-dicyclohexylcarbodiimide (DCC) in

Scheme 4



18: R_1 = H, R_2 = Me, R_3 = C_6H_5
(or **19**: R_1 = Et, R_2 = H, R_3 = C_6H_{11})

20: R_1 = H, R_2 = Me, R_3 = C_6H_5
(or **21**: R_1 = Et, R_2 = H, R_3 = C_6H_{11})

presence of catalytic amount of 4-dimethylaminopyridine (DMAP) in CH_2Cl_2:DMF (1:1) at 0°C (for **8**), or at -10°C (for **9**). Little epimerization(~5-10%) could not be avoided in the case of **9**. The minor isomer could easily be separated by standard column chromatography.

The coupled ester **18** (or **19**) was also a mixture of diastereomers (for silyl-protected hemiketal moiety)[21] and rotamers. It was not very stable at higher temperature and, thus, ^{1}H NMR spectrum could not be run at elevated temperatures. It showed correct mass in LSIMS. Though the diastereomers could be separated here, they were used as a mixture in the next step. The methanolic solution of the ester was hydrogenated under atmospheric pressure using Pd-C as catalyst. The resulting amino acid was dissolved in dry ethanol-free chloroform (0.002M) and stirred under argon with benzotriazol-1-yloxy tris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent, 5 molar equivalents) and DIPEA (7 molar equivalents) was added drop by drop. The liberated amine underwent smooth macrolactamization leading to the formation of cyclized product **20** (or **21**). The product which showed correct mass in LSIMS was, once again, used as a mixture of diastereomers in the final desilylation step. Treatment of a THF solution of **20** (or **21**) with HF-pyridine led to the formation of the desired RAP-P **5** (or **6**). It was an equilibrating mixture of isomers (ca 3:1) which, although, could be separated by standard column chromatography or RP-HPLC, were equilibrating back, once in solution, to the same mixture. This was also proved by running a 2D-TLC which showed 4 spots as expected. The two isomers were, probably, 6 and 7-membered hemiketals.

The binding affinity of **5** for FKBP12 was measured and compared to those of ascomycin and rapamycin using a competitive binding assay developed at Sandoz[16]. The IC50 values for **5**, ascomycin, and rapamycin in this assay were found to be 9.6, 0.52 and 0.45 nM, respectively. Though it exhibited very powerful binding with FKBP12, unlike rapamycin, it showed no activity in IL-6 dependent B-cell proliferation and, in contrast to FK506, showed no activity in the IL-2 reporter assay. The affinity of **6** for FKBP12, its IL-6 dependent B-cell proliferation assay and IL-2 reporter assay are currently under study.

In conclusion, a highly practical protocol is developed which will allow to generate a large number of FKBP12-binding ligands with or without immunosuppressive activity. It is conceivable that molecules may emerge from such libraries of hybrid compounds that mimic or antagonize the biological actions of rapamycin or FK506/ascomycin, or even both. Such compounds may find useful applications in biology as tools, or in medicine as improved immunosuppressive agents.

Experimental Section

General Procedures - NMR spectra were recorded on Varian Gemini 200, Varian Unity 400, Bruker WH 360 and Bruker AMX 500 instruments. IR spectra were recorded on Shimadzu IR-470. MS were recorded on VG MICROMASS 70-70H and VG Auto Spec M Spectrometers under electron impact (EI), chemical ionization (CI) or liquid secondary ion mass spectrometric (LSIMS) techniques.

All reactions were monitored by thin layer chromatography (TLC) carried out on 0.25 mm E.Merck silica gel plates (60F-254) with UV light, I_2, 7% ethanolic phosphomolybdic acid-heat and 2.5% ethanolic

anisaldehyde (with 1% AcOH and 3.3% conc. H_2SO_4)-heat as developing agents.

All reactions were carried out under nitrogen atmosphere with dry, freshly distilled solvents under anhydrous conditions unless otherwise noted. Yields refer to chromatographically and spectroscopically homogeneous materials unless otherwise noted.

Methyl carbinol 11. To a solution of methyl keto compound **10**[19] (0.489 g, 1.0 mmol) in dry THF (4 mL) under nitrogen atmosphere at -78°C, Li-selectride (1.5 mL, 2M solution, 3.0 mmol) was added drop by drop. After stirring for 15 minutes at the same temperature it was quenched with saturated NH_4Cl solution (10 mL) and brought to room temperature. The aqueous layer was extracted with ethyl acetate (2x10 mL). The combined organic extracts were washed with brine (10 mL), dried (Na_2SO_4) and concentrated in vacuo. Column chromatography (SiO_2, 20-40% EtOAc in petroleum ether eluant) gave pure methyl carbinol **11** (0.471 g, 96%) as a syrupy liquid. R_f= 0.2 (silica, 50% EtOAc in petroleum ether). ^{1}H NMR (360 MHz, DMSO-d_6, 150°C, single conformer): δ 7.40-7.26 (m, 5H, aromatic), 5.57 (br s, 1H, O<u>H</u>), 5.18 (AB q, 2H, OC<u>H</u>$_2$Ph), 5.15 (d, J=9 Hz, 1H, C2-<u>H</u>), 4.90 (br, 1H, O<u>H</u>), 4.0 (m, 1H, C14-<u>H</u>), 3.75-3.6 (m, 2H, C17-<u>H</u>, C16-<u>H</u>), 3.28 (s, 3H, OC<u>H</u>$_3$), 3.10 (m, 2H, C6-<u>H</u>), 2.20-1.20 (m, 13H, C<u>H</u>$_2$ and C<u>H</u>), 1.01 (d, J=6.3 Hz, 3H, C17-C<u>H</u>$_3$), 0.79 (d, J=6.7 Hz, 3H, C11-C<u>H</u>$_3$). MS(LSIMS): calcd for $C_{26}H_{37}NO_8$ (M^+): 491, found m/z 514 (M^++Na), 492 (M^++H), 474 (M^++H-H_2O).

Hemiketal-protected compound 7. To a solution of methyl carbinol **11** (0.393 g, 0.8 mmol) and 2,6-lutidine (372 μL, 3.2 mmol) in CH_2Cl_2 (4 mL) at 0°C was added TMSOTf (319 μL, 1.76 mmol). After stirring for 15 minutes at the same temperature, the reaction mixture was diluted with ether (20mL), washed with brine (2x10 mL), dried (Na_2SO_4) and concentrated in vacuo. The residue was taken in THF (10 mL) and 0.4 mL of 0.1N HCl was added to it. After stirring for 15 minutes at room temperature, the reaction mixture was diluted with ether (20 mL), washed with 10% $NaHCO_3$ (10 mL), brine (10 mL), dried (Na_2SO_4) and concentrated in vacuo. Purification by column chromatography (SiO_2, 20-35% EtOAc in petroleum ether eluant) afforded the hemiketal-protected derivative **7** (0.414 g, 92%) as a syrupy liquid. R_f=0.3 (silica, 50% EtOAc in petroleum ether). ^{1}H NMR (360 MHz, DMSO-d_6, 150°C, mixture of two diastereomers): δ 7.3 (m, 5H, aromatic), 5.18 (AB q, 2H, OC<u>H</u>$_2$Ph), 5.15 (two d, J=9 Hz, 1H, C2-<u>H</u>), 3.86 (m, 1H, C14-<u>H</u>), 3.75-3.60 (m, 2H, C17-<u>H</u>, C16-<u>H</u>), 3.29 and 3.28 (two s, 3H total, OC<u>H</u>$_3$), 3.10 (m, 2H, C6-<u>H</u>), 2.20-1.20 (m, 13H, C<u>H</u>$_2$ and C<u>H</u>), 1.02 and 1.01 (two d, J=6.5 Hz, 3H total, C17-C<u>H</u>$_3$), 0.73 (d, J=6.7 Hz, 3H, C11-C<u>H</u>$_3$), 0.15 (s, 9H, Si<u>Me</u>$_3$). MS(LSIMS): calcd for $C_{29}H_{45}NO_8Si$(M^+): 563, found m/z 586 (M^++Na), 564 (M^++H).

Cbz-D-homoPhe-Gly-Sar-OMe (14). To a solution of Cbz-Gly-Sar-OMe (**12**) (0.294 g, 1.0 mmol) in MeOH (2 mL) was added dil. HCl (1.1 mL, 1N solution) and Pd-C (50 mg). It was hydrogenated under atmospheric pressure using a H_2-balloon for 0.5 h. The reaction mixture was then filtered through a short pad of celite and the filter cake washed with MeOH (2x2 mL). The combined filtrate was concentrated in vacuo and the residue azeotroped with dry toluene (5 mL) to afford syrupy amine hydrochloride.

In a separate RB flask Cbz-D-homoPhe-OH **13** (0.313 g, 1.0 mmol) was dissolved in dry amine-free DMF (1 mL) and treated sequentially with HOBt (0.135 g, 1.0 mmol) and EDCI (0.192 g, 1.0 mmol) at

0°C. After stirring for 15 minutes at 0°C, the amine hydrochloride, prepared above and taken in dry amine-free DMF (1 mL) was added to it followed by the dropwise addition of DIPEA (171 μL, 1.0 mmol). Two more batches of DIPEA (each of 85 μL, 0.5 mmol) were added in 1h intervals. The reaction mixture was left stirring at room temperature for 12h. It was then diluted with EtOAc (10 mL) and washed with brine (2x5 mL), dried (Na_2SO_4) and concentrated in vacuo. Purification by column chromatography (SiO_2, EtOAc eluant) gave the tripeptide **14** (0.387 g, 85%) as an amorphous white solid. R_f=0.6 (silica, EtOAc). ^1H NMR (200 MHz, $CDCl_3$): δ 7.4-7.0 (m, 10H, aromatic), 7.05 (m, 1H, Gly-NH), 5.63 (m, 1H, homoPhe-NH), 5.07 (AB q, 2H, OCH_2Ph), 4.3 (m, 1H, homoPheα-H), 4.1 (s, 2H, Saraα-H), 4.1-3.85 (m, 2H, Glyα-H), 3.71 (s, 3H, OCH_3), 3.01 (s, 3H, NCH_3), 2.65 (t, J=7.6 Hz, 2H, $PhCH_2CH_2$), 2.25-1.85 (m, 2H, $PhCH_2CH_2$). MS(LSIMS): calcd for $C_{24}H_{29}N_3O_6(M^+)$: 455, found m/z 478 (M^++Na), 456 (M^++H).

Cbz-D-homoPhe-Gly-Sar-OH (8). To a solution of the tripeptide methyl ester **14** (0.364 g, 0.8 mmol) in THF-MeOH-H_2O (3:1:1, 10 mL) at 0°C was added solid LiOH (0.058 g, 2.4 mmol). After stirring for 1h at room temperature, it was quenched with dil.HCl (1N, ~2.5 mL) to bring the pH to ~ 1-2. THF and MeOH were removed under vacuum and the aqueous layer was extracted with EtOAc (2x5 mL). The EtOAc extracts were washed with brine (5 mL), dried (Na_2SO_4), concentrated in vacuo and the residue azeotroped with dry toluene (5 mL) to get the acid **8** (0.353 g, quantitative) as a white amorphous solid which was used directly in the next step.

Cbz-D-γ-cHexAbu-Gly-D-Abu-OMe (17). Hydrogenation of Cbz-Gly-D-Abu-OMe **15** (0.308 g, 1.0 mmol) was carried out in the same way as described above to get the amine hydrochloride H_3N^+-Gly-D-Abu-OMe. ^-Cl which was coupled with Cbz-D-γ -cHexAbu-OH[22](**16**) (0.319 g, 1.0 mmol) to afford the tripeptide **17** (0.389 g, 82%) as a white amorphous solid. R_f= 0.45 (silica, 70% EtOAc in petroleum ether). ^1H NMR (200 MHz, $CDCl_3$) : δ7.40-7.30 (m, 5H, aromatic), 6.80 (m, 2H, Gly-NH and Abu-NH), 5.27 (d, J=6.8 Hz, 1H, γ-cHexAbu-NH), 5.95 (AB q, 2H, OCH_2Ph), 4.52 (m, 1H, Abuα-H), 4.1 (m, 2H, γ-cHexAbuα-H and Glyα-H), 3.85 (dd, J=16.8, 5.0 Hz, 1H, Glyα-H'), 3.74 (s, 3H, CO_2CH_3), 2.0-1.5, 1.5-1.0 (m, 15H, CH_2, CH), 0.89 (m, 2H, cHex-CH), 0.89 (t, J=7.5 Hz, 3H, Abu-CH_3), HRMS(EI): calcd for $C_{25}H_{37}N_3O_6(M^+)$: 475.2682, found m/z 475.2710.

Cbz-D-γ-cHexAbu-Gly-D-Abu-OH (9). The hydrolysis of the methyl ester **17** (0.38 g, 0.8 mmol) with LiOH (0.058 g, 2.4 mmol) in THF-MeOH-H_2O (3:1:1, 10 mL) was done in the same way as described above for **8** to get the acid **9** (0.37 g, quantitative) as a white amorphous solid which was used directly in the next step.

The coupled ester 18. To a solution of the hemiketal-protected methyl carbinol 7 (0.197 g, 0.35 mmol), the tripeptide acid **8** (0.309 g, 0.7 mmol) and DMAP (0.0085 g, 0.07 mmol) in CH_2Cl_2-DMF (1:1, 2 mL) was added DCC (0.144 g, 0.7 mmol) at 0°C under nitrogen atmosphere. Stirring was continued at the same temperature for 24h. It was then diluted with EtOAC (10 mL), filtered through a sintered funnel and the filter cake washed with EtOAc (2x2 mL). The filtrate and washings were combined and washed with brine (5 mL), dried (Na_2SO_4) and concentrated in vacuo. Column chromatography (SiO_2, 30-60% EtOAc in petroleum ether eluant) gave the ester **18** (0.293 g, 85%) as a white amorphous solid. R_f= 0.85 (silica, 70%

EtOAc in petroleum ether). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.42-7.08 (m, 15H, aromatic), 6.9 (m, 1H, Gly-NH), 5.5-5.0 (m, 7H, homoPhe-NH, OCH_2Ph, C2-H, C17-H), 4.3-3.7, 3.4-3.0 and 3.0-2.8 (m, 9H, Glyα-H, Sarα-H, homoPheα-H, C16-H, C14-H, C6-H), 3.22 (s, 3H, OCH_3), 2.93 (s, 3H, N-CH_3), 2.55 (t, J=7.9 Hz, 2H, CH_2Ph), 2.20 (m, 2H, $PhCH_2$-CH_2), 1.85 (m, 1H, C11-H), 1.8-1.3 (m, 12H, CH_2), 1.18 (d, J=6.67 Hz, 3H, C17-CH_3), 0.77 (d, J=6.6 Hz, 3H, C11-CH_3), 0.18 and 0.1 (two s, total 9H, $Si(CH_3)_3$). MS(LSIMS): calcd for $C_{52}H_{70}N_4O_{13}Si(M^+)$: 986, found m/z 1009($M^+$+Na), 986($M^+$).

The coupled ester 19. The second tripeptide acid **9** (0.323 g, 0.7 mmol) was esterified with the methylcarbinol **7** (0.197 g, 0.35 mmol) using DCC (0.144 g, 0.7 mmol) and DMAP (0.0085 g, 0.07 mmol) in the same as way as described above for **18**. Only this time the DCC was added at -10°C and reaction continued at the same temperature for 48h. Column chromatography (SiO_2, 30-60% EtOAc in petroleum ether eluant) afforded pure **19** (0.275 g, 78%) as a white amorphous solid. R_f= 0.5 (silica, 70% EtOAc in petroleum ether). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.4-7.23 (m, 10H, aromatic), 7.02 (m, 2H, Gly-NH, Abu-NH), 5.44 (m, 1H, C17-H), 5.24-4.95 (m, 6H, OCH_2Ph, C2-H, γ-cHexAbu-NH), 4.54(q, J=6.0 Hz, 1H, γ-cHexAbuα-H), 4.12 (dd, J=16.8, 1.7 Hz, 1H, Glyα-H), 4.08 (q, J=7.0 Hz, 1H, Abuα-H), 3.84 (dd, J=16.8, 4.6 Hz, 1H, Glyα-H'), 3.35, 3.3 (two s, total 3H, OCH_3), 4.45-3.24 (m, 4H, C16-H, C14-H, C6-H), 2.28 (m, 1H, C3-H), 2.24 (m, 1H, C11-H), 2.0-0.8 (m, 31H, CH_2, CH), 0.85 (t, J=6.9 Hz, 3H, Abu-CH_3), 0.78 and 0.77 (two d, J=6 Hz, 3H, C11-CH_3), 0.2-0.02 (four s, total 9H, $Si(CH_3)_3$. MS(LSIMS): calcd for $C_{53}H_{78}O_{13}N_4Si(M^+)$: 1006, found m/z 1029($M^+$+Na), 1007 ($M^+$+H).

TMS-Protected Cyclic Product 20. The coupled product **18** (98.6 mg, 0.1 mmol) was dissolved in MeOH (5 mL) and Pd-C (20 mg) was added to it. It was then hydrogenated under atmospheric pressure using a H_2-balloon for 1h. The reaction mixture was then filtered through a short pad of celite and filter cake washed with MeOH (2x2 mL). The filtrate and washings were combined and concentrated in vacuo. The residue was azeotroped with dry toluene (5 mL) and dried under vacuum. It was then taken in dry ethanol-free $CHCl_3$ and BOP reagent (0.221 g, 0.5 mmol) was added to it followed by dropwise addition of DIPEA (120 μL, 0.7 mmol). After stirring for 12h at room temperature it was concentrated in vacuo, the residue dissolved in EtOAc (10 mL), washed with 0.1N HCl (5 mL), brine (5 mL), dried (Na_2SO_4) and concentrated. Column chromatography (SiO_2, 40-70% EtOAc in petroleum ether eluant) gave the cyclic product **20** (33 mg, 45%) as a mixture of diastereomers, which was used directly in the next step. ^{1}H NMR (400 MHz, $CDCl_3$, mixture of isomers, ca.3:2): δ 7.28-7.16 (m, 5H, aromatic), 6.93 and 6.70 (two d, J= 7.8 Hz, 1H, homoPheNH), 6.71 and 6.42 (two m, 1H, Gly-NH) 5.14 and 5.07 (two m, 1H, C17-H), 5.07 and 5.0 (two m, 1H, C2-H), 4.52, 4.12, 3.84 and 3.69 (four d, J= 17.8 Hz, 2H, Sarα-H), 4.52 (m, 1H, homoPheα-H), 4.37 and 4.24 (two dd, J= 6 and 17.8 Hz, 1H, Glyα-H), 3.98-3.78 (m, 2H, C16-H, Glyα-H'), 3.56 (m, 1H, C14-H), 3.37 and 3.34 (two s, 3H, OCH_3), 3.22 and 3.10-2.94 (m, 2H, C6-H), 2.99 and 2.95 (two s, 3H, NCH_3), 2.66 (q, J= 8.2 Hz,2H, $PhCH_2$), 2.4-1.2 (m, 15H, CH_2, and CH), 1.17 and 1.15 (two d, J= 6.9 Hz, 3H, C17-CH_3), 0.83 (d, J= 6.5 Hz, 3H, C11-CH_3), 0.2 (s, 9H, $Si(CH_3)_3$). MS(LSIMS): calcd for $C_{37}H_{56}N_4O_{10}Si(M^+)$: 744, found m/z 745($M^+$+H).

TMS-Protected Cyclic Product 21. The coupled product **19** (100.6 mg, 0.1 mmol) was subjected to

hydrogenation and macrolactamization following the same method as described above for **20** to get the cyclic product **21** (32 mg, 42%) as a mixture of diastereomers, which was used directly in the next step. ^{1}H NMR (400 MHz, $CDCl_3$, major isomer): δ6.97 (m, 1H, GlyNH), 6.28 (d, J= 8.3 Hz, 1H, cHexAbuNH), 6.19 (d, J= 8.0 Hz, 1H, AbuNH), 5.15 (m, 1H, C17-H), 5.10 (m, 1H, C2-H), 4.38 (m, 2H, cHexAbuα-H, C14-H), 4.05 (dd, J= 7.2 and 16.0 Hz, 1H, Glyα-H), 3.9 (m, 1H, C16-H), 3.58 (dd, J= 5.3 and 16.0 Hz, 1H, Glyα-H'), 3.46 (q, J= 8.0 Hz, 1H, Abuα-H), 3.3 (s, 3H, OCH_3), 3.12 and 3.03 (m, 2H, C6-H), 2.44 - 1.10 (m, 28H, CH_2 and CH), 1.18 (d, J= 7.0 Hz, 3H, C17-CH_3), 0.94 (t, J= 7.0 Hz, 3H, Abu-CH_3), 0.85 (m, 2H, cHex-CH), 0.8 (d, J= 6.5 Hz, 3H, C11-CH_3), 0.2 (s, 9H, $Si(CH_3)_3$). MS(LSIMS): calcd for $C_{38}H_{64}N_4O_{10}Si(M^+)$: 764, found m/z 765($M^+$+H).

RAP-P 5. The TMS-protected compound **20** (30 mg, 40 μmol) was dissolved in dry THF (1 mL) in a plastic vial and treated with HF- pyridine (100 μL). After TLC analysis revealed complete desilylation (24h), the reaction mixture was quenched with aqueous saturated $NaHCO_3$ (5 mL) and extracted with EtOAc (3x10 mL). The combined organic layers were washed with brine (1x10 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Column chromatography (SiO_2, 80-100% EtOAc in petroleum ether eluant) gave pure **5** (25 mg, 92%) as a white amorphous solid. **5**: mixture of isomers in DMSO (ca.3:1) at room temperature. R_f= 0.12 (major), 0.10 (minor) (silica, EtOAc). High Pressure Liquid Chromatography(HPLC) analytical, Lichrospher 100, RP-18, 125x4 mm, ***5*** μM column): Retention time (RT) (min), 10.08 (major), 9.2 (minor) (gradient, 70:30 to 30:70 H_2O: CH_3CN in 20 min., flow rate 1.5 mL/min., at 40°C). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of isomers, ~ 3:1): δ8.11 (major, dd, J=4.8 Hz) and 7.73 (minor, dd, J=6.6, 3 Hz), (total 1H, GlyNH), 7.4 (minor, d, J=7.8 Hz) and 6.67 (major, d, J=7.8 Hz), (total 1H, homoPheNH), 7.3-7.1 (m, 5H, aromatic), 6.73 (major, s) and 6.57 (minor, s), (total 1H, C10-OH), 5.04 (major, dq, J=6.6, 3 Hz) and 4.92 (minor, dq, J=6.6 Hz) (total 1H, C17-H), 4.96 (m, 1H, C2-H), 4.49 (major, d, J=18 Hz) and 3.86 (minor, d, J=16.8 Hz) (total 1H, Sarα-H), 4.32 (m, 2H, C20-H and C16-H), 4.14 (major, m) and 3.85 (minor, m) (total 2H, Glyα-H), 3.95 (m, 2H, Sarα-H' and C14-H), 3.54 (major, br d, J=15 Hz) and 3.47 (minor, br d, J=15 Hz) (total 1H, C6-H), 3.39 (major, dd, J=15 Hz) and 3.31 (minor, dd, J=15 Hz) (total 1H, C6-H'), 3.21 (major, s) and 3.19 (minor, s) (total 3H, OCH_3), 3.04 (minor, s) and 2.77 (major, s) (total 3H, SarNCH_3), 2.58 (m, 2H, PhCH_2), 2.2-1.2 (m, 15H, CH_2 and CH), 1.11 (major, d, J=6.6 Hz) and 1.0 (minor, d, J=6.6 Hz) (total 3H, C17-CH_3), 0.73 (d, J=6.6 Hz, 3H, C11-CH_3). MS(LSIMS): calcd for $C_{34}H_{48}N_4O_{10}$ (M^+): 672, found m/z 679(M^++Li).

RAP-P6. Desilylation of compound **21** (26.7 mg, 35 mol) was carried out in the same way as described above which was followed by chromatographic purification to get **6** (22 mg, 90%) as a white amorphous solid. R_f= 0.1 (silica, EtOAc). ^{1}H NMR (400 MHz, DMSO-d_6, major isomer) : δ 8.3 (m, 1H, Gly-NH) 7.74 (d, J=6.4 Hz, 1H, Abu-NH), 7.4 (d, J=7.6 Hz, 1H, γ-cHexAbu-NH), 6.53 (s, 1H, C10-OH), 5.08 (m, 1H, C17-H), 4.95 (m, 1H, C14-H), 4.88 (d, J=5.0 Hz, 1H, C2-H), 4.35 (m, 1H, γ-cHexAbuα-H), 4.08 (q, J=6.4 Hz, 1H, Abuα-H), 3.96 (m, 1H, C16-H), 3.85 (dd, J=16, 6.4 Hz, 1H, Glyα-H), 3.6 (dd, J=16, 5 Hz, 1H, Glyα-H'), 3.35 (s, 3H, OCH_3), 3.5-3.1 (m, 2H, C6-H), 2.1 (m, 1H, C3-H), 2.0 (m, 1H, C11-H), 1.9-1.4, 1.2-1.0 (m, 26H, CH_2, CH), 1.05 (d, J=6.6 Hz, 3H, C17-CH_3), 0.85 (t, J=7 Hz, 3H, Abu-CH_3), 0.85(m, 2H,

cHex-C<u>H</u>), 0.73 (d, J=6.6 Hz, 3H, C11-C<u>H</u>$_3$). MS(LSIMS): calcd for $C_{35}H_{56}N_4O_{10}$ (M^+): 692, found m/z 715(M^++Na, 100%), 693 (M^++H, 8%), 675 (M^++H-H_2O,57%).

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