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SOLID PHASE SYNTHESIS OF A BIASED MINI TETRAPEPTOID-LIBRARY FOR THE DISCOVERY OF MONODENTATE ITAM MIMICS AS ZAP-70 INHIBITORS

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Abstract: The biased library was composed of a novel phosphotyrosine mimic fixed in the P1 position of a tetrapeptoid and combined with three lipophilic N-substituents at the remaining positions giving a total of 27 single compounds. Screening for ZAP-70 antagonism identified **8** as a novel selective monodentate ZAP-70 antagonist and lead in the search for new immunosuppressive drugs.

Introduction

Protein phosphorylation is a primary mechanism of cellular signal transduction. Upon ligand binding, a number of cell surface receptors undergo phosphorylation on specific tyrosyl residues within their intracellular domains. The phosphorylated segments in turn participate in subsequent signal transduction through association with specific src homolgy region 2 (SH2) domains (1). For example, on ligand binding, the intracellular portion of the T cell receptor (TCR) is phosphorylated on two tyrosine residues within the immunoreceptor tyrosine activated motifs (ITAMs), which provides binding sites for the downstream signalling protein ZAP-70. The tandem SH2 domains of ZAP-70 associate with the doubly phosphorylated ITAMs, which contain sequences of 16-17 amino acids $YXX(L/I)X_{7\text{-}8}YXX(L/I)$ where X is variable. The binding of ZAP-70 to the TCR is believed to be essential for signal transduction, as peptides that block the association of ZAP-70 with the ITAMs also inhibit T-cell signalling events (2). Therefore, ZAP-70 is an attractive target for the development of novel immunosuppressive drugs.

Design and Synthesis

The recent X-ray crystal structure (3) of the tandem SH2 domains of human ZAP-70 in complex with a doubly tyrosine phosphorylated 19-meric peptide derived from the ITAM of the TCR provides a basis for antagonist-design. The major bindig energy between ITAM and ZAP-70 derives from the bidentate interaction of both phosphotyrosines (pY) and their lipophilic residues in pY+3 with the tandem SH2 domains of ZAP-70; the correct distance between both pYs being more important than the specific sequence between them. Monophosphorylated (monodentate) ITAM-based peptides show affinities 100-1000 times lower than the bidentate analogs with their entropic advantage. Our approach to the design of ZAP-70 antagonists neglects the entropic factor in favor of increased cell-permeability by incorporating on a tetrapeptoid skeleton only one pY mimic accompanied by three lipophilic substituents (Ethyl, Isopropyl or 3-Phenylpropyl). The foreseeable lower affinity of our monodentate ZAP-70 antagonists in comparison to

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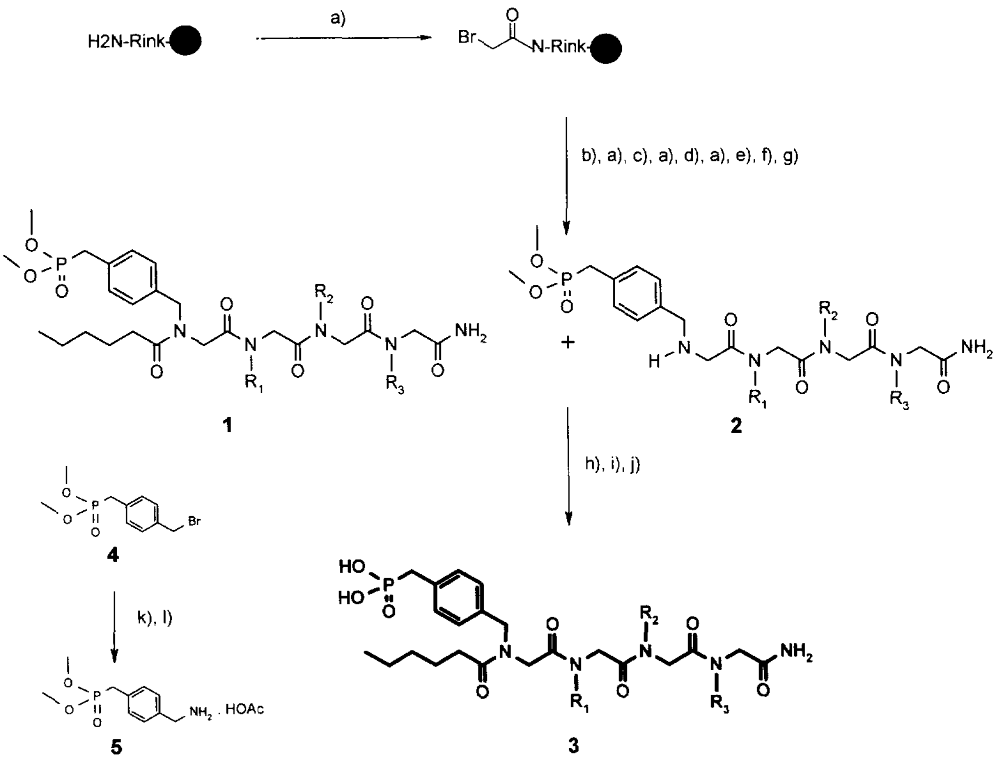
bidentates is expected to be compensated *in vivo* by the improved absorption of the peptoid skeleton carrying *only one* charged phosponate group. In contrast to peptides with phosphono(difluoro)methylphenylalanine as pY mimetics, peptoids with the our pY mimetic are expected to display a higher resistance to proteases (4). Here we report the solid phase synthesis (Scheme 1) of a mini-library of 27 single compounds containing a new non-hydrolyzable pY mimetic prepared in high purity on Rink amide MBHA resin. The peptoids were assembled on the resin as dimethylphosphonates using known protocols (5), then removed from the resin, analyzed by HPLC and filtered over silica gel. Silica gel filtration was mandatory, because acylation with hexanoic acid / PyBroP proceeded only in ca. 50% yield. The unreacted basic benzylamine derivatives **2** were readily separated (SiO_2; CH_2Cl_2 / MeOH 98:2 to 95:5) from the less polar **1**, which in the last step of the synthesis were hydrolyzed by TMSBr to the desired phosphonic acids **3** and analyzed by HPLC. The end-products **3** were obtained in amounts of 20-100mg with purities >85% (10).

Scheme 1

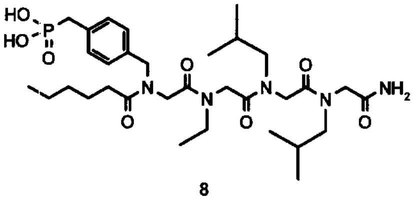


a) $BrCH_2COOH$, DIC, DMF, 0°C to r.t., 2.5h b) R_3-NH_2 (30 eq.) DMSO, r.t., 12h. c) R_2-NH_2 (30 eq.) DMSO, r.t., 12h. d) R_1-NH_2 (30 eq.) DMSO, r.t., 12h. e) Dimethylphosphonomethylbenzylamine **5** (10 eq.) DMSO, r.t., 12h. f) CH_2Cl_2, 3x Hexanoic acid, PyBroP, DIEA, r.t.; each 2h. g) Cleavage from resin (TFA/H2O 95/5 7min r.t.) h) HPLC-analysis, filtration over silica gel, HPLC-analysis. i) TMS-Br, CH_2Cl_2, 0°C to r.t., 2h. j) Acetone/water, RT, 2.5h. k) NaN_3, DMF/water 13:1, 3h, 95%. l) PPh3, ether/water 1:1, 18h r.t., 95%

Dimethylphosphonomethylbenzylamine **5** was prepared from bromide **4** (9), which was converted in high yield to the corresponding azide; reduction of the latter with triphenylphosphine gave **5** (Scheme 1). **5** was preferably stored as its acetate salt.

Results and discussion

All measurements were performed using the BIAcore®2000 optical biosensor (Biacore AB) (6). Table 1 summarizes the SH2-inhibitory properties of the mini-library. Only peptoid **8** out of the 27 tetrapeptoids proved to be a ZAP-70 inhibitor. In comparison with the ITAM-derived 19-mer peptide NQLPpYNELNLGRREEpYDVLD (IC_{50}=30nM) the IC_{50}=25µM of **8** is in the expected range due to the lack of the entropically favorable bidentate-effect.



8

Table 1

	R_1	R_2	R_3	LCK (-%)*	Grb2 (-%)*	ZAP-70	
						(-%)*	IC_{50}(µM)
7	Isobutyl	Isobutyl	Isobutyl	3	6	16	
8	Ethyl	Isobutyl	Isobutyl	6	8	92	[redacted]
9	Phpropyl**	Isobutyl	Isobutyl	16	0	5	
10	Isobutyl	Ethyl	Isobutyl	9	30	61	100
11	Ethyl	Ethyl	Isobutyl	2	2	14	
12	Phpropyl	Ethyl	Isobutyl	7	3	4	
13	Isobutyl	Phpropyl	Isobutyl	1	3	4	
14	Ethyl	Phpropyl	Isobutyl	3	4	8	
15	Phpropyl	Phpropyl	Isobutyl	6	3	16	
16	Isobutyl	Isobutyl	Ethyl	1	4	16	
17	Ethyl	Isobutyl	Ethyl	0	2	17	
18	Phpropyl	Isobutyl	Ethyl	5	6	15	
19	Isobutyl	Ethyl	Ethyl	61	15	97	63
20	Ethyl	Ethyl	Ethyl	4	6	9	
21	Phpropyl	Ethyl	Ethyl	4	5	0	
22	Isobutyl	Phpropyl	Ethyl	4	5	5	
23	Ethyl	Phpropyl	Ethyl	7	8	56	100
24	Phpropyl	Phpropyl	Ethyl	4	4	16	
25	Isobutyl	Isobutyl	Phpropyl	1	5	12	
26	Ethyl	Isobutyl	Phpropyl	15	2	9	
27	Phpropyl	Isobutyl	Phpropyl	32	32	10	>100
28	Isobutyl	Ethyl	Phpropyl	10	2	20	
29	Ethyl	Ethyl	Phpropyl	1	4	3	
30	Phpropyl	Ethyl	Phpropyl	4	20	38	>100
31	Isobutyl	Phpropyl	Phpropyl	3	0	10	
32	Ethyl	Phpropyl	Phpropyl	0	2	24	
33	Phpropyl	Phpropyl	Phpropyl	4	5	34	100

*) Inhibition at 100µM **) Phpropyl: 3-Phenylpropyl

ZAP-70 showed a preference for the isobutyl group in R_3, which mimics Leu in pY+3 of the ITAMs; low affinity was observed for the ethyl group in R_3 and none for the 3-phenylpropyl group. The latter substituent appears too large in contrast to the ethyl group, which in **10** and **19** still showed modest activity. **8** is remarkably specific and has no affinity towards SH2-domains of LCK or Grb2. Currently, work is in progress towards the preparation of follow-up libraries aiming at more potent monodentate and bidentate ZAP-70 inhibitors by optimizing the peptoid-substituents R_1-R_3

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(6) *Biomolecular interaction analysis (BIA)*- All measurements were performed using the BIAcore®2000 optical biosensor (Biacore AB). Streptavidin was covalently coupled to CM5-sensorchips by derivatizing the carboxymethylated dextran-hydrogel on the chip surface with N-ethyl-N'-(dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide; thus the signal increased by 800-1000 so-called resonance units (RU). Unreacted groups were quenched during exposure to ethanolamine. Phosphopetides, N-terminally modified by biotinyl-aminocaproic acid, were then immobilized on the sensor chips to a level of 30-40 RU. The peptide sequences were EPQpYEEI (for LCK; IC50: 30 nM), DSPpYVNVQ (for GRB2; IC50: 40 nM), NQLPpYNELNLGRREEpYDVLD (for ZAP-70; IC50: 30 nM).SH2 domains of LCK, GRB2 and the tandem domains of ZAP-70 were cloned and expressed in E.coli as fusion proteins to glutathione-S-transferase (GST), and then purified as described (7, 8). Proteins were injected in buffer A (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 0.5 mM DTT, 0.005 % (v/v) Tween 20) at a concentration of 50 nM, and at a flow rate of 10 µl/min for 4 min. Association phase binding reached an equilibrium of 400-800 RU in the absence of antagonist. Inhibitors were titrated from 10^{-7} to 10^{-4} M. Seven seconds into the dissociation phase, monitored for 2 min in free buffer flow, report points were taken to generate dose-response curves for estimating IC50 values; ie., the inhibitor concentrations causing a signal decrease of 50 percent. The sensorchip surfaces were very stable and could after sample injection be regenerated using 0.05 % (w/v) SDS.

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(10) The correct structure of all compounds was confirmed by MS- and NMR-spectra. Analytical HPLC was performed on a Merck-Hitachi system with a reversed-phase HPLC column (25 cm x 4.6 cm) and a gradient elution (H_2O/CH_3CN/TFA 70:30:0.05 to H_2O/CH_3CN/TFA 50/50/0.05 within 15 min. at 60^0C, 1 ml/min.) Under these conditions, **8** had a retention time of 9.2 min.