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Communication

**Search for new synthetic immunosuppressants II.
Tetrazole analogues of hymenistatin I[★]✉**

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Linear and cyclic hymenistatin I (HS I) analogues with dipeptide segments Ile²-Pro³, Pro³-Pro⁴ and Val⁶-Pro⁷ replaced by their tetrazole analogues Ile²-Ψ[CN₄]-Ala³, Pro³-Ψ[CN₄]-Ala⁴ and Val⁶-Ψ[CN₄]-Ala⁷ were synthesized by the solid phase peptide synthesis method and cyclized with the TBTU and/or HATU reagent. The peptides were examined for their immunosuppressive activity in the lymphocyte proliferation test (LPT).

A serious problem of transplantology, a dynamically expanding branch of contemporary medicine, is survival of the transplanted organ. A wider use of two existing and effective immunosuppressants, cyclosporin A (CsA,

sandimmun[®]) [1] and FK-506 [2], as potent drugs for the prevention of graft rejection is limited by their side effects. In this situation, the search for new immunosuppressants exhibiting a similar mechanism of action but de-

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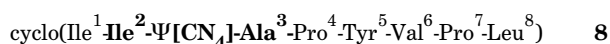
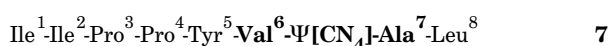
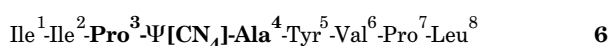
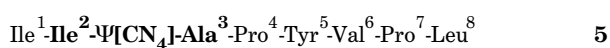
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Abbreviations: ESI-MS, electrospray ionization mass spectroscopy; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HS I, hymenistatin I; LPT, lymphocyte proliferation test; PHA, phytohemagglutinin; SPPS, solid phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate.

void of toxicity, especially in the group of naturally existing immunomodulatory peptides and their analogues, has become an important goal for medicinal chemistry.

Hymenistatin I [HS I] is a natural cyclic octapeptide of the sequence cyclo(Ile¹-Ile²-Pro³-Pro⁴-Tyr⁵-Val⁶-Pro⁷-Leu⁸) isolated by Petit *et al.*, in 1990 [3]. It has been shown by Siemion and coworkers [4] that hymenistatin I demonstrates some immunosuppressive activity in respect to both humoral (PFC) and cellular (DTH) immune response. A characteristic feature of this compound is the presence of a *cis*-amide bond between Pro³-Pro⁴ residues, a β Vla turn in the Ile²-Tyr⁵ region and a β I or β II turn in the Val⁶-Ile¹ region in CHCl₃ and Me₂SO solution, respectively [3, 5].

To evaluate the significance of the Ile²-Pro³, Pro³-Pro⁴ and Val⁶-Pro⁷ peptide bonds for HS I biological activity, we have synthesized three linear and three cyclic hymenistatine I analogues, with the dipeptide segments Ile²-Pro³, Pro³-Pro⁴ and Val⁶-Pro⁷ replaced by their tetrazole analogues Ile²- Ψ [CN₄]-Ala³, Pro³- Ψ [CN₄]-Ala⁴ and Val⁶- Ψ [CN₄]-Ala⁷, respectively.



The 1,5-disubstituted tetrazole ring, Ψ [CN₄], has been proposed as a surrogate for *cis*-amide bonds, making it a valuable tool in the design of conformationally constrained peptidic receptor probes [6–10]. Since native hymenistatin I has been demonstrated to have only weak biological activity which precludes its use as an immunosuppressant we decided to look for immunosuppressive activity of hymenistatin I analogues, employing a standard test used for the assessment of immunosuppressive activity.

MATERIALS AND METHODS

The synthesis of desired peptides c(Ile²- Ψ [CN₄]-Ala³)HS I **8**, c(Pro³- Ψ [CN₄]-Ala⁴)HS I **9** and c(Val⁶- Ψ [CN₄]-Ala⁷)HS I **10** as well as the native compound was achieved by fragment condensation on a polymeric support (Merrifield type resin) using TBTU or HATU as a coupling reagent. The synthetic strategy is shown for peptide **8** as an example (Fig. 2). The tetrazole building blocks **4** was synthesized separately in solution (Fig. 1, Table 1) following a previously reported strategy [8, 9] and were used in the solid phase peptide synthesis procedure. The known proclivity of the C-terminal α -carbon of tetrazole dipep-

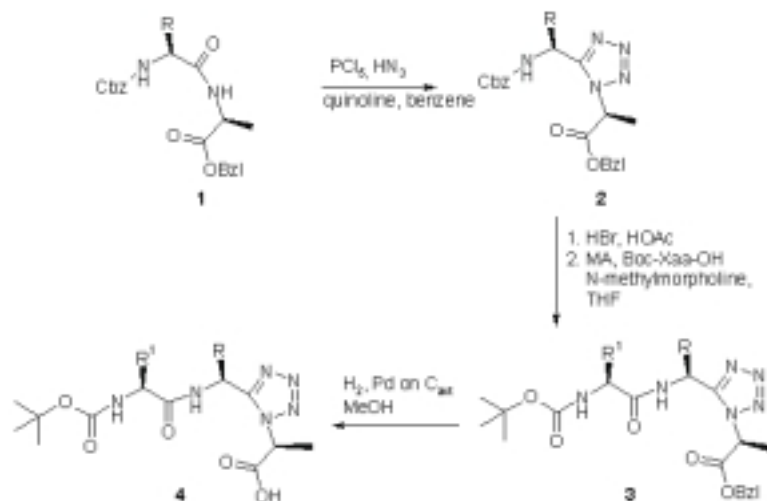


Figure 1. Scheme of tetrazole tripeptides synthesis.

Table 1. Analytical data for tetrazole tripeptides

Compound	Yield [%]	$[\alpha]_D$ (c = 1, MeOH)	t_R	R_f^c	M_r^d calc./found
Boc-Ile-Ile-Ψ[CN ₄]-Ala-OBzl	69.9	-69.4	16.42 ^a	0.56 ¹	514.6/515.1
Boc-Ile-Pro-Ψ[CN ₄]-Ala-OBzl	79.8	-17.9	12.21 ^a	0.53 ¹	498.6/498.9
Boc-Tyr(2,6-diCl)-Val-Ψ[CN ₄]-Ala-Obzl	68.2	-53.7	17.5 ^a	0.64 ¹	697.6/698.2
Boc-Ile-Ile-Ψ[CN ₄]-Ala-OH	95.2	-54.8	5.46 ^b	0.64 ²	424.5/424.8
Boc-Ile-Pro-Ψ[CN ₄]-Ala-OH	98.1	-16.2	5.62 ^b	0.58 ²	408.4/409.1
Boc-Tyr(2,6-diCl)-Val-Ψ[CN ₄]-Ala-OH	96.4	-52.3	6.21 ^b	0.68 ²	607.4/607.9

^aAnalytical HPLC with linear gradient 50–70% B/25 min. (A) 0.05% trifluoroacetic acid in water, and (B) 0.038% trifluoroacetic acid in acetonitrile/H₂O (90:10, v/v); ^bgradient 40–60% B/25 min. ^cTLC systems: 1 = dichloromethane/acetone (30:1, v/v); 2 = butanol/acetic acid/water (4:1:1, by vol.). ^dRelative molecular mass calculated/found by FAB-MS measurements.

tides to epimerize [7–9] on prolonged base exposure prompted us to omit the neutralization step in the solid phase synthesis of peptides containing the tetrazole unit.

The cyclization of linear precursors was performed in solution using TBTU and/or HATU as a coupling reagent (Fig. 2). The structure of all peptides was confirmed by ESI-MS (Table 2).

All compounds demonstrated sufficient solubility and were tested for immunosuppressive activity by the lymphocyte proliferation test (LPT). Lymphocytes were isolated from pe-

ripheral blood of healthy volunteers. Cells were stimulated with phytohemagglutinin (PHA) and incubated with different concentrations of the agents under study or cyclosporin A. Incorporation of [³H]thymidine, which was added to the cultures 24 h before cell harvesting, was determined in a liquid scintillation counter and expressed as counts per minute (c.p.m.).

RESULTS AND DISCUSSION

All synthesized analogues did not affect lymphocyte proliferation, thus are devoid of immunosuppressive activity as assayed by the LPT (Table 3). It was very surprising that even native hymenistatin I did not affect lymphocyte proliferation in this kind of biological test. On the other hand, our study does not exclude that the examined compounds could have an immunomodulatory effect on other immunological functions. In fact it has previously been noted that the native compound demonstrated some immunosuppressive activity in animal models (mice). However, the relevance of the biological activity of native hymenistatin I described in animal models by Cebrat *et al.* [4] to effective immunosuppression in humans is not clear and the lack of effect of the native compound on cytokine (IL-2 or IL-6) generation makes its clinical potential use unlikely. Unfortunately, as demonstrated

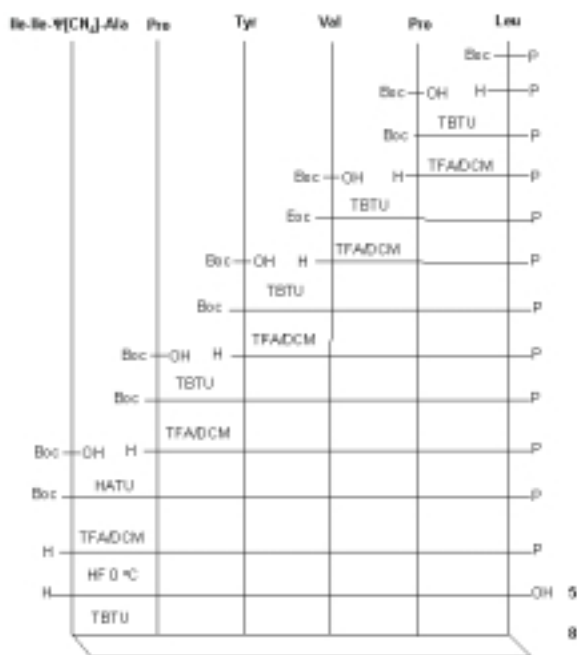


Figure 2. Scheme of c(Ile²-Ψ[CN₄]-Ala³)HS I synthesis.

Table 2. Analytical data for hymenistatin I analogues

Peptide	Yield ^a (%)	t_R^b (min)	M_r^c calc./found
5 Linear (Ile ² -Ψ[CN ₄]-Ala ³)HS I	83.1	6.37	910.07/909.87
6 Linear (Pro ³ -Ψ[CN ₄]-Ala ⁴)HS I	90.4	9.22	910.07/910.01
7 Linear (Val ⁶ -Ψ[CN ₄]-Ala ⁷)HS I	81.2	6.18	910.07/909.92
8 c(Ile ² -Ψ[CN ₄]-Ala ³)HS I	24.4	10.86	892.06/892.02
9 c(Pro ³ -Ψ[CN ₄]-Ala ⁴)HS I	23.8	14.03	892.06/891.96
10 c(Val ⁶ -Ψ[CN ₄]-Ala ⁷)HS I	21.3	10.14	892.06/892.08

^aPeptide content in crude product as shown by analytical HPLC with linear gradient 30–90% B/25 min. (A) 0.05% trifluoroacetic acid in water, and (B) 0.038% trifluoroacetic acid in acetonitrile/H₂O (90:10, v/v); ^bgradient as above; ^crelative molecular mass calculated/found by ESI-MS measurements.

Table 3. Effect of the compounds studied on lymphocyte proliferation

Compound studied	Control solution (PHA 10 µg/ml)	2.5 µg/ml	5 µg/ml	10 µg/ml	20 µg/ml
Linear (Ile ² -Ψ[CN ₄]-Ala ³)HS I	172505	178933	169858	168503	165870
Linear (Pro ³ -Ψ[CN ₄]-Ala ⁴)HS I	172505	186371	188707	179547	187384
Linear (Val ⁶ -Ψ[CN ₄]-Ala ⁷)HS I	172505	177748	166299	154650	163309
c(Ile ² -Ψ[CN ₄]-Ala ³)HS I	200864	207386	199482	215386	227716
c(Pro ³ -Ψ[CN ₄]-Ala ⁴)HS I	200864	206011	213888	207746	215366
c(Val ⁶ -Ψ[CN ₄]-Ala ⁷)HS I	200864	207154	202170	212090	220303
Native HS I	200864	204662	211458	217956	224532

Results are shown in c.p.m. (counts per minute)

in our study, modification of hymenistatin I by a 1,5-disubstituted tetrazole ring did not

improve its immunosuppressive activity *in vitro* to any significant degree.

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