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Synthesis of 4-amino-3-oxo-tetrahydroazepino[3,4-*b*]indoles: new conformationally constrained Trp analogs

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Abstract—The synthesis of tryptophan analogs is reported in which the conformation has been constrained by formation of a seven-membered lactam. Boc-protected 2′-formyl tryptophan was obtained by SeO_2 oxidation of Boc-tetrahydro-β-carboline-3-carboxylic acid. Reductive amination was performed with a variety of amines and amino acid esters using sodium cyanoborohydride, followed by ring closure to the target compounds. The constrained Trp derivative has been incorporated into the endomorphin-1 opioid peptide sequence to probe the bioactive conformation.

1. Introduction

Aromatic amino acids (Phe, Tyr, Trp, and His) are often a key element of bioactive peptides. The conformational restriction of such residues has been a successful strategy to increase the potency, selectivity, and metabolic stability of peptides, and to influence their agonist/antagonist properties.[1–8] The concept of topographical control or the control of χ-space has been used to design many constrained analogs of these natural amino acids.[1–23] One of the most successfully applied analogs is that in which the α-nitrogen has been linked to the aromatic ring through a methylene unit, resulting in the six-membered ring derivatives tetrahydroisoquinoline-3-carboxylic acid (**1**, Tic), tetrahydro-β-carboline-3-carboxylic acid (**2**, Tcc) or spinacine (**3**, Spi), which limit the side chain conformation to a χ^1 of $-60°$ or $+60°$.[9] These have found many applications in peptides[1,4,5,7,9–11] as well as in peptide mimetics.[12–17] This widespread use was certainly helped by the easy availability of the homochiral compounds (Fig. 1).

An alternative conformational constraint involves the fixation of the side chain conformation to a χ^1 of $+60°$ or $180°$ by linking the aromatic ring to the nitrogen atom of the succeeding amino acid, resulting in the 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (**4**, Aba (R′=H), Hba (R′=OH)) skeleton.[3,5] This type of Phe or Tyr analog has been applied successfully in the design of peptides with improved pharmacological properties and of selective protease inhibitors.[5,7,18–21] We have also published a solid-supported synthesis allowing a parallel synthesis of 2,4-di-substituted analogs of this drug-like molecule.[22] We now report an efficient synthetic route toward the Trp analog 4-amino-3-oxo-3,4,5,10-tetrahydro-1*H*-azepino[3,4-*b*]indol-2-yl acetic acid (**5**, Aia). Only one report of this heterocycle has been found in the literature, where it is part of a polycyclic structure having ACE inhibition properties (**6**).[23,24] We also report the use of the Aia residue to evaluate the proposed

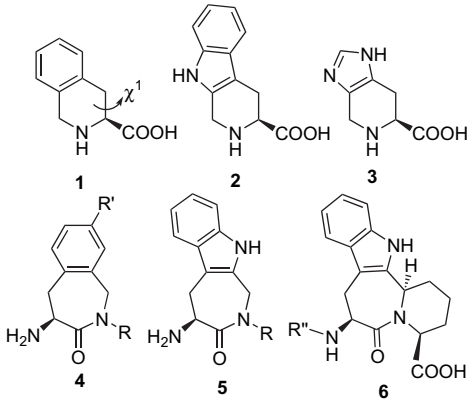

Figure 1. Conformationally constrained analogs of Phe, Trp, and His.

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bioactive conformation of the opioid peptide endomorphin-1. Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) **7** is a highly potent and selective endogenous agonist of the μ-receptor. A model for its bioactive conformation with the Trp side chain in the *gauche* (+) or trans conformation was proposed using NMR spectroscopy and molecular modeling.[25] Since these are the χ^1 values allowed by the Aia structure, the bioactivity of [Aia]endomorphin-1 can either confirm or disprove the proposal.

2. Results and discussion

The conformation of Ac-Aia-Phe-NH_2 **8** was studied by molecular modeling using MacroModel, as a model for the C-terminus of endomorphin-1. The results confirmed that the low energy conformations for **8** have a trans χ^1 conformation for the Aia ring. All low energy conformations are extended structures, which are in agreement with our previous studies on the benzazepine analog Ac-Aba-Gly-NHMe.[26] These also indicated that despite the fact that similar dehydro-lactams were reported as β-turn mimics,[27] the benzazepinones prefer extended conformations. Most of the conformations of **8** have a *gauche* (−) orientation of the Phe side chain. An extended conformation with the ^{3}Trp side chain in the *gauche* (+) and the ^{4}Phe side chain in the trans conformation has been proposed as the bioactive conformation of endomorphin-1.[25] The conformation of **8** having a trans conformation of the Phe side chain has an energy of 13.9 kJ/mol above the minimum and showed the best backbone similarity with the proposed bioactive conformation of endomorphin-1. A superimposition of both conformations is shown in Figure 2.

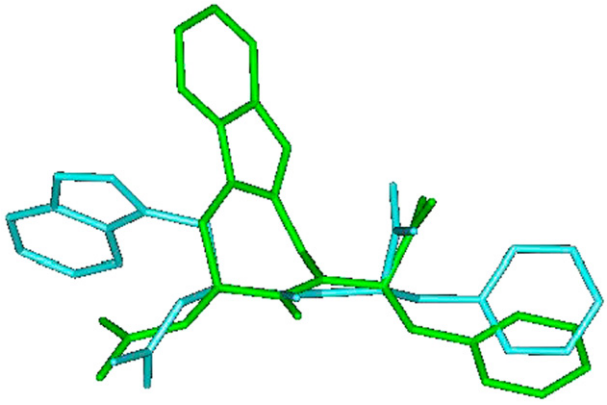
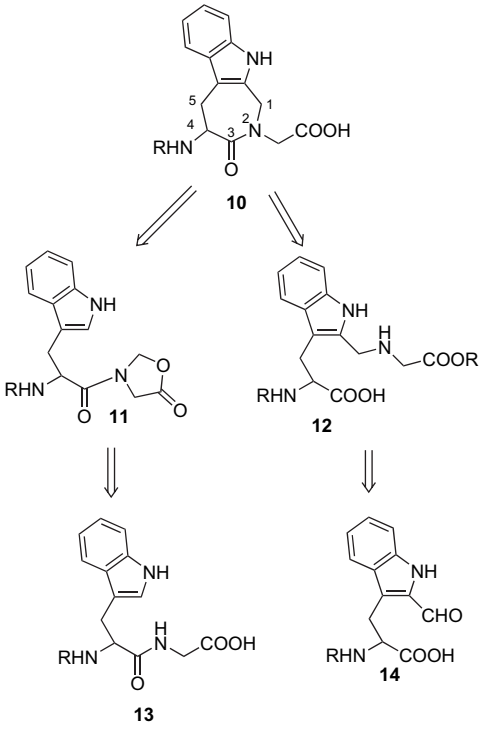


Figure 2. Superimposition of the low energy trans-conformation for the C-terminus of endomorphin-1 as reconstructed from Podlogar et al.[25] and Ac-Aia-Phe-NH_2 **8**.

A maintained or an increased binding affinity of [Aia]endomorphin-1 **9** is not in agreement with the bioactive model, while a decreased binding affinity can be ascribed to a different orientation of the indole ring, since the conformation of the remaining part of the molecule is very similar, including the positioning of the carboxamide, which is claimed to be responsible for the μ-selectivity.[25]

Two general synthetic routes have been described for the synthesis of 4-amino-tetrahydro-2-benzazepin-3-ones derived from Phe and Tyr: one involves the cyclization of an *N*-acyliminium ion, which is generated from an enamide[23,24] or from an oxazolidinone precursor,[8,28,29] the other proceeds through lactam formation after reductive amination of *N*-protected *o*-formyl phenylalanine.[30,31] In Scheme 1, these synthetic routes are shown for the Trp derivatives. However, all our attempts to prepare the oxazolidinone of phthaloyl-protected Trp-Gly **11** failed. We therefore focused our attention on the preparation of *N*-protected 2′-formyl tryptophan **14**.

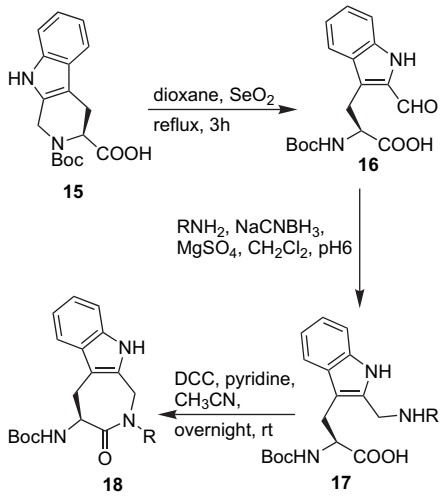


Scheme 1. Retrosynthetic pathways for 4-amino-azepino[3,4-*b*]indoles.

Although the formation of the 2′-formyl derivative by direct formylation of *N*,*N*-dimethyl-homotryptamine under Vilsmeier conditions was reported,[32] the formylation of Pht-Trp-OMe resulted in only 15% of the desired 2′-formyl compound, the main product being the 1′-formyl isomer. The SeO_2 oxidation of *N*-acetyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid methyl ester was reported to give *N*-acetyl-2′-formyl Trp-OMe in 52% yield.[33] We have found that this oxidation reaction does not require the ester protective group, and that the yield was better with Boc-nitrogen protection (76%, Scheme 2). As a result, the oxidation of Boc-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid **15**[34] resulted in Boc-2′-formyl tryptophan **16**, which could be isolated by precipitation, and has an optimal protection for further transformation.

The reductive amination of **16** was performed with various amines or amino acid esters using $NaCNBH_3$. Intermediates **17** were not isolated,[31] but the crude mixtures were used in the following cyclization reaction using DCC as the coupling agent. After overnight stirring, the cyclization was completed.

The crude products **18** were purified by flash chromatography. The isolated yields were calculated over two steps



Scheme 2. Synthesis of 4-amino-azepino[3,4-*b*]indoles.

from **16** and are reported in Table 1. The optical purity of the final compounds was studied by preparing (*S*)- and (*R*)-**18b**, starting from L- and D-Trp, respectively. After Boc-deprotection, the enantiomers were derivatized with Marfey's reagent[35] and separated by RP-HPLC. The individual enantiomers showed less than 1% racemization.

Table 1. Yields of amino-indoloazepinones **18** after flash chromatography

Entry	Yield (%)
18a R=$CH_2C_6H_5$	48
18b R=$CH_2COOCH_2CH_3$	42
18c R=$CH_2COOCH_2C_6H_5$	45
18d R=(*S*)$CH(CH_3)COOCH_3$	61
18e R=(*S*)$CH(CH_3)COOCH_2C_6H_5$	53
18f R=(*S*)$(CH_2)_5COOCH_3$	54
18g R=(*S*)$CH(CH_2C_6H_5)COOCH_3$	31
18h R=$CH(CH_3)_2$	22
18i R=$CH_2CH(CH_3)_2$	38
18j R=$CH_2CH_2CH_2CH_3$	28
18k R=cyclohexyl	24

In our previous studies on the preparation of 4-amino-tetrahydro-2-benzazepin-3-ones, we used solid-supported cyanoborohydride for the reductive amination and supported carbodiimide for the cyclization, in order to facilitate isolation of the target compounds.[22] Reductive aminations of **16** with several amines were carried out in the presence of solid-supported cyanoborohydride. However, the reaction times required for the completion of these reactions were exceedingly long (between 8 and 13 days). The resulting secondary amines **17h–k** were cyclized using DCC in solution, and the obtained yields and purities of the cyclic compounds **18h–k** were lower than those of **18a–g**, which were obtained from the solution phase synthesis.

We have incorporated the constrained dipeptide **18g** into the endomorphin-1 sequence. The synthesis of the tetrapeptide was performed in solution. Boc-Aia-Phe-OMe **18g** was Boc-deprotected using 1 M HCl–EtOAc and coupled to Boc-Tyr-Pro using TBTU as the coupling reagent. The resulting peptide (Boc-Tyr-Pro-Aia-Phe-OMe **19**) was treated with saturated ammonia in MeOH or 0.58 M LiOH (5 equiv) to give **9** and **21**, respectively. These peptides were purified by semi-preparative HPLC.

The receptor affinities of the peptide analogs for the μ- and δ-opioid receptors were measured in rat brain membranes[36] and are summarized in Table 2.

Table 2. Receptor binding affinities of the endomorphin-1 analogs

Peptide sequence	IC_{50} μ±SEM (nM)[a]	IC_{50} δ±SEM (nM)[b]
Tyr-Pro-Trp-Phe-NH_2 **7**	9.7±2.21	1600±7.2
Tyr-Pro-Trp-Phe-OH **20**	1023.8±3.03	1300±6.8
Tyr-Pro-Aia-Phe-NH_2 **9**	223.8±3.64	$>10^4$
Tyr-Pro-Aia-Phe-OH **21**	$>10^4$	$>10^4$

[a] μ-Ligand:[^{3}H]naloxone.
[b] δ-Ligand:[^{3}H][^{5,6}Ile]deltorphin.

Both analogs showed a substantial loss in μ-affinity compared to the native μ-selective endomorphin-1 sequence **7** or to the non-selective C-terminal acid analog **20**. No affinity for the δ-opioid receptor was observed. The most active analog **9** was tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and mouse vas deferens (MVD) as reported in detail elsewhere.[37] This [Aia]endomorphin-1 analog showed to be a full agonist in both assays with IC_{50}=299±22 nM and IC_{50}=678±83 nM. The GPI contains μ- and κ-receptors, whereas the MVD contains mainly δ-receptors, although μ- and κ-receptors are also present in this tissue.[37] This may explain the agonist activity of **9** in the GPI assay, despite the low δ-affinity of the compound.

The conformation of Ac-Aia-Phe-NH_2 **8** was studied as a model for the C-terminus of endomorphin-1 by NMR spectroscopy in DMSO-d_6 solution. The large temperature dependence of the NH_2 signals indicates that there is no formation of a turn conformation having an intramolecular hydrogen bond (Table 3). The H^{α} and H^{β} coupling constants are consistent with a trans χ^1 conformation for the Aia ring. This is in complete agreement with the molecular modeling results.

Table 3. NMR study of Ac-Aia-Phe-NH_2 **8**

	δ ($CDCl_3$, ppm)	δ (DMSO, ppm)	Δδ (ppm)	Δδ/Δ*T* (ppb/K)
Ac-N*H*	7.0	8.1	1.1	−4.7
NH_2	6.1	7.1	1.0	−4.3
NH_2	5.9	6.9	1.0	−4.3

These results indicate that the conformational constraints imposed by this type of fixation of the ^{3}Trp residue are not well tolerated by the μ-opioid receptor. Figure 2 indicates that the decreased binding affinity of the [Aia]endomorphin-1 **9** can be ascribed to an orientation of the indole ring that is not well accepted by the receptor. Nevertheless, analog **9** was able to fully activate the μ-receptor.

3. Conclusions

We have developed a suitable synthetic procedure giving an easy access to the hitherto largely unexplored homochiral 4-amino-3-oxo-tetrahydroazepino[3,4-*b*]indole skeleton. The procedure allows the introduction of a variety of substituents at the ring nitrogen. After Boc-deprotection, further derivatization of 4-amino group is possible. This will be very important to generate new leads in medicinal chemistry.[22] Moreover we have demonstrated that the dipeptide analog

can be introduced into the bioactive peptides using standard methodology and that these peptide analogs can provide interesting information about the receptor-bound conformation of Trp-containing peptides. We are currently further exploring the potential of this building block in peptide mimicry.

4. Experimental section

4.1. General

RP-HPLC was performed using an Agilent 1100 Series system (Waldbronn, Germany) with a Discovery BIO Wide Pore C18 column (ID=0.46 cm, *L*=25 cm, PS=5 μm, Bellefonte, PA, USA). Gradient 1 or 2 was used (grad. 1: *t*=0 min, 97% A, 3% B; *t*=30 min, 3% A, 97% B. Grad. 2: *t*=0 min, 97% A, 3% B; *t*=20 min, 3% A, 97% B), flow rate: 1 mL/min, λ=215 nm. The mobile phases (water and acetonitrile) contained 0.1% TFA. Semi-preparative HPLC was performed using a Merck Hitachi with DAD detector and RP C-18 column (Discovery BIO Wide Pore C18, ID=1.0 cm, *L*=25 cm, PS=5 μm), gradient: *t*=0 min, 97% A, 3% B; *t*=15 min, 70% A, 30% B; *t*=30 min, 60% A, 40% B, flow rate: 3 mL/min, λ=215 nm. The mobile phases (water and acetonitrile) contained 0.05% TFA. TLC analysis was performed on a plastic sheet precoated with silica gel $60F_{254}$ (Merck). Silica gel 60 (0.04–0.063 mm) from Merck was used for flash chromatography. ^1H and ^{13}C NMR spectra were recorded on an AC 250 Bruker spectrometer at 250 and 63 MHz or on a Varian Unity Plus spectrometer at 200 and 50 MHz in $CDCl_3$ or DMSO-d_6. Mass spectra were recorded on a VG Quattro II spectrometer using electrospray ionization (positive ion mode). High-resolution mass spectra were recorded on a LCT TOF spectrometer. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. Infrared spectral data were obtained using an Avatar 370 FTIR. Receptor binding and functional assays were performed as described previously.[36,37] 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid was synthesized from L-Trp and formaldehyde using the Pictet–Spengler reaction.[34] The Boc-protected compound was obtained according to the standard procedure[38] in a mixture of dioxane–water.

4.1.1. 2(*S*)-*N*-(*tert*-Butoxycarbonyl)-2′-formyl tryptophan 16. To a stirred solution of Boc-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1.00 g, 3.16 mmol) in dioxane (25 mL), selenium dioxide (0.457 g, 4.11 mmol) was added. The reaction mixture was refluxed for 3 h (monitored with TLC, sprayed with 2,4-dinitrophenylhydrazine to give orange spots). After filtration of the hot mixture through dicalite and the removal of the solvent, the residue was redissolved in methanol and filtered through filter paper. The filtrate was evaporated and the product was precipitated from $CHCl_3$ to give a yellow solid (0.80 g, 76%). $[\alpha]_D^{20}$ +21.9 (*c* 1, CH_3OH); IR (neat): 3328, 2979, 1683, 1649, 1164, 741; HPLC (grad. 1): t_R=17.3 min; R_f ($CHCl_3$–MeOH–AcOH 9:1:0.1): 0.44; MS: 215 [M−Boc−H_2O]$^+$, 233 [M−Boc]$^+$, 277 [M−*t*Bu]$^+$, 333 [M+H]$^+$, 665 [2M+H]$^+$; ^1H NMR (250 MHz, DMSO-d_6): δ 1.26 (s, 9H), 3.16–3.74 (m, 2H), 4.19 (br s, 1H), 7.09–7.64 (m, 4H), 7.76 (s, 1H), 9.96 (s, 1H), 11.68 (s, 1H); ^{13}C NMR (63 MHz, DMSO-d_6): δ 25.8 (CH_2), 28.4 (CH_3), 44.7 (CH_2), 55.0 (CH), 78.50 (C), 113.1–137.9 (C+CH), 155.6 (C), 173.4 (C), 182.5 (C); HRMS (ESI$^+$) exact mass calcd for $C_{17}H_{20}N_2O_5Na$ [M+H]$^+$: 355.1270, found: 355.1279.

4.2. General procedure for the synthesis of 4-amino-3-oxo-tetrahydroazepino[3,4-*b*]indoles 18a–g

Aldehyde **16** (0.332 g, 1 mmol) was suspended in CH_2Cl_2 (puriss. p.a. 15 mL) and amino acid ester (hydrochloric or *p*-toluenesulfonic acid salt, 1.05 mmol) was added. The pH was adjusted to 6 with acetic acid or *N*-methyl-morpholine, followed by the addition of $MgSO_4$ (20 wt %) and $NaCNBH_3$ (2.5 mmol). The reaction was monitored with HPLC or TLC. When the reaction was completed (typically 3 h) the solvent was evaporated and the residue was redissolved in acetonitrile (80 mL). Pyridine (2 mmol) was added and the reaction mixture was cooled to 0 °C for 10 min and DCC (1.1 mmol) was added. After 1 h of stirring at 0 °C the reaction was continued overnight at room temperature. Oxalic acid (5 M solution in DMF, 2.5 mL) was added and after 30 min of stirring the precipitate was filtered off. The filtrate was evaporated and the redissolved in EtOAc (100 mL) then washed with 1 M HCl (3×30 mL), saturated aqueous $NaHCO_3$ (3×30 mL), and brine (3×20 mL). The organic layer was dried over $MgSO_4$. After evaporation there was a small amount of remaining *N*,*N*'-dicyclohexylurea (DCU). The crude product was dissolved in small amount of CH_2Cl_2 and DCU was filtered off. The solvent was evaporated and the product was purified by flash column chromatography (CH_2Cl_2–EtOAc).

4.2.1. 2(*S*)-*tert*-Butyl 2-(benzyl)-3-oxo-1,2,3,4,5,10-hexahydroazepino[3,4-*b*]indol-4-yl carbamate 18a. Yield: 48%; $[\alpha]_D^{20}$ +54.1 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3293, 2974, 1694, 1641, 1161, 731; HPLC (grad. 1): t_R=23.5 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.82; MS: 306 [M−Boc]$^+$, 350 [M−*t*Bu]$^+$, 406 [M+H]$^+$, 428 [M+Na]$^+$, 444 [M+K]$^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.51 (s, 9H), 2.86 (pseudo-t, 1H), 3.29 (pseudo-d, 1H), 3.88 (d, *J*=17.25 Hz, 1H), 4.48 (d, *J*=15.25 Hz, 1H), 4.83 (d, *J*=18.25 Hz, 1H), 4.84 (d, *J*=15 Hz, 1H), 5.02–5.12 (m, 1H), 6.02 (d, *J*=7.25 Hz), 7.00–7.33 (m, 9H), 8.09 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 28.9 (CH_3), 29.5 (CH_2), 44.7 (CH_2), 51.4 (CH), 51.9 (CH_2), 80.2 (C), 109.8–141.7 (C+CH), 155.7 (C), 173.2 (C); HRMS (ESI$^+$) exact mass calculated for $C_{24}H_{27}N_3O_3Na$ [M+Na]$^+$: 428.1950, found: 428.1960.

4.2.2. 2(*S*)-Ethyl [4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]acetate 18b. Yield 42%; $[\alpha]_D^{20}$ +106.7 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3325, 2978, 1710, 1649, 1162, 738; HPLC (grad. 1): t_R=20.5 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.55; MS: 302 [M−Boc]$^+$, 346 [M−*t*Bu]$^+$, 402 [M+H]$^+$, 424 [M+Na]$^+$, 440 [M+K]$^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.11 (t, *J*=7.13 Hz, 3H), 1.50 (s, 9H), 2.77 (pseudo-t, 1H), 3.22 (pseudo-d, 1H), 3.98 (d, *J*=17.25 Hz, 1H), 4.02 (d, *J*=17.75 Hz, 1H), 4.07 (k, *J*=7 Hz, 2H), 4.45 (d, *J*=17.5 Hz, 1H), 5.00–5.07 (m, 1H), 5.14 (d, *J*=17.5 Hz, 1H), 5.94 (d, *J*=7 Hz, 1H), 6.97–7.26 (m, 4H), 8.45 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 14.3 (CH_3), 28.8 (CH_3), 29.4 (CH_2), 46.9 (CH_2), 50.7 (CH_2), 51.4 (CH), 61.9 (CH_2), 80.3 (C), 109.5–135.1 (C+CH), 155.6 (C), 169.4 (C), 173.4 (C); HRMS (ESI$^+$) exact mass calculated for $C_{21}H_{27}N_3O_5Na$ [M+Na]$^+$: 424.1848, found: 424.1832.

4.2.3. 2(*S*)-Benzyl [4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-acetate 18c. Yield 45%; $[\alpha]_D^{20}$ +74.0 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3315, 2975, 1707, 1649, 1164, 738; HPLC (grad. 1): t_R=24.36 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.7; MS: 364 $[M-Boc]^+$, 408 $[M-tBu]^+$, 464 $[M+H]^+$, 486 $[M+Na]^+$, 502 $[M+K]^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.49 (s, 9H), 2.75 (m, 1H), 3.23 (pseudo-d, 1H), 3.96 (d, *J*=17 Hz, 1H), 4.09 (d, *J*=17.5 Hz, 1H), 4.47 (d, *J*=17.5 Hz, 1H), 4.99 (m, 1H), 5.05 (s, 2H), 5.13 (d, *J*=17.5 Hz, 1H), 5.92 (d, *J*=7 Hz, 1H), 6.99–7.27 (m, 9H), 8.30 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 29.3 (CH_3), 30.1 (CH_2), 46.9 (CH_2), 50.7 (CH_2), 51.4 (CH_2), 67.6 (CH_2), 80.2 (C), 118.7–135.4 (C+CH), 155.6 (C), 169.2 (C), 173.5 (C); HRMS (ESI^+) exact mass calculated for $C_{26}H_{29}N_3O_5Na$ $[M+Na]^+$: 486.2005, found: 486.1981.

4.2.4. 2(*S*)-Methyl 2-[4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-propanoate 18d. Yield 61%; $[\alpha]_D^{20}$ +57.0 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3341, 2979, 1701, 1646, 1165, 735; HPLC (grad. 1): t_R=21.02 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.65; MS: 302 $[M-Boc]^+$, 346 $[M-tBu]^+$, 402 $[M+H]^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.41 (d, *J*=7 Hz, 3H), 1.49 (s, 9H), 2.86 (pseudo-t, 1H), 3.27 (s, 3H), 3.33 (pseudo-d, 1H), 4.17 (d, *J*=17.25 Hz, 1H), 4.94 (d, *J*=16.75 Hz, 1H), 5.08–5.17 (m, 1H), 5.33 (k, *J*=7.25 Hz, 1H), 5.95 (d, *J*=7.25 Hz, 1H), 7.02–7.37 (m, 4H), 8.32 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): 15.3 (CH_3), 28.8 (CH_3), 29.5 (CH_2), 41.4 (CH_2), 51.4 (CH), 52.5 (CH_3), 52.7 (CH), 80.2 (C), 109.5–135.1 (C+CH), 155.6 (C), 172.3 (C), 173.1 (C); HRMS (ESI^+) exact mass calculated for $C_{21}H_{27}N_3O_5Na$ $[M+Na]^+$: 424.1848, found: 424.1849.

4.2.5. 2(*S*)-Benzyl 2-[4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-propanoate 18e. Yield 53%; $[\alpha]_D^{20}$ +62.0 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3259, 2978, 1712, 1647, 1150, 734; HPLC (grad. 1): t_R=26.27 min; R_f ($CHCl_3$–EtOAc 3:1): 0.4; MS: 378 $[M-Boc]^+$, 422 $[M-tBu]^+$, 478 $[M+H]^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.42 (d, *J*=7.18 Hz, 3H), 1.47 (s, 9H), 2.77 (pseudo-t, 1H), 3.23–3.31 (m, 1H), 4.14 (d, *J*=16.9 Hz, 1H), 4.58 (d, *J*=12.13 Hz, 1H), 4.83 (d, *J*=12.13 Hz, 1H), 4.88 (d, *J*=16.9 Hz, 1H), 5.03–5.12 (m, 1H), 5.37–5.45 (m, 1H), 5.93 (d, *J*=7.28 Hz, 1H), 7.01–7.37 (m, 9H), 8.13 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 15.2 (CH_3), 28.5 (CH_3), 29.4 (CH_2), 41.4 (CH_2), 51.5 (CH), 52.8 (CH), 67.5, 80.2 (C), 111.3–135.1 (C+CH), 155.6 (C), 171.7 (C), 173.0 (C); HRMS (ESI^+) exact mass calculated for $C_{27}H_{31}N_3O_5Na$ $[M+Na]^+$: 500.2161, found: 500.2153.

4.2.6. 2(*S*)-Methyl 6-[4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-hexanoate 18f. Yield 54%; $[\alpha]_D^{20}$ +91.1 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3315, 2931, 1711, 1639, 1156, 736; HPLC (grad. 1): t_R=20.86 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.58; MS: 344 $[M-Boc]^+$, 388 $[M-tBu]^+$, 444 $[M+H]^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.05–1.49 (m, 15H), 2.18 (t, *J*=7 Hz, 2H), 2.78 (pseudo-t, 1H), 3.22–3.37 (m, 2H), 3.48–3.62 (m, 4H), 3.98 (d, *J*=17.25 Hz, 1H), 4.97–5.04 (m, *J*=16.25 Hz, 2H), 5.98 (d, *J*=7 Hz, 1H), 7.00–7.33 (m, 4H), 8.62 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 24.7 (CH_2), 26.5 (CH_2), 28.1 (CH_2), 28.8 (CH_3), 29.4 (CH_2), 34.1 (CH_2), 45.6 (CH_2), 48.9 (CH_2), 51.3 (CH), 51.9 (CH_3), 80.1 (C), 109.7–135.1 (C+CH), 155.6 (C), 172.6 (C), 174.6 (C); HRMS (ESI^+) exact mass calculated for $C_{24}H_{33}N_3O_5Na$ $[M+Na]^+$: 466.2318, found: 466.2328.

4.2.7. 2(*S*)-Methyl 2-[4-[(*tert*-butoxycarbonyl)amino]-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-3-phenylpropanoate 18g. Yield 31%; $[\alpha]_D^{20}$ +36.4 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3332, 2976, 1708, 1646, 1163, 735; HPLC (grad. 1): t_R=24.51 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.72; MS: 378 $[M-Boc]^+$, 422 $[M-tBu]^+$, 478 $[M+H]^+$, 500 $[M+Na]^+$, 516 $[M+K]^+$; ^1H NMR (250 MHz, $CDCl_3$): δ 1.47 (s, 9H), 2.80 (pseudo-t, 1H), 3.06 (dd, 1H), 3.30 (d, 2H), 3.34 (s, 3H), 4.1 (d, *J*=17.75 Hz, 1H), 4.92 (d, *J*=17 Hz, 1H), 4.98–5.06 (m, 1H), 5.28 (k, *J*=6.25 Hz, 1H), 5.87 (d, *J*=7.25 Hz, 1H), 6.93–7.35 (m, 9H), 7.85 (s, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ 28.8 (CH_3), 29.4 (CH_2), 35.8 (CH_2), 43.2 (CH_2), 51.3 (CH), 52.6 (CH_3), 60.1 (CH), 80.1 (C), 109.6–136.7 (C+CH), 155.5 (C), 171.2 (C), 173 (C); HRMS (ESI^+) exact mass calculated for $C_{27}H_{31}N_3O_5Na$ $[M+Na]^+$: 500.2161, found: 500.2139.

4.3. General procedure for the reductive amination with solid-supported cyanoborohydride 18h–k

Aldehyde **16** (0.332 g, 1 mmol) and amine (1.2 mmol) were dissolved in CH_2Cl_2 (10 mL). Pivalic acid (10 mmol) and polymer-bound cyanoborohydride (0.581 g, 2.5 mmol, loading=4.3 mmol/g) were added. When the reaction was finished (between 8 and 13 days), polymer-bound benzaldehyde (0.167 g, 0.5 mmol, loading=3 mmol/g) was added to scavenge the excess of primary amine. Scavenging took 12 days. The resin was filtered off and the filtrate was evaporated. Cyclization of products **17h–k** was performed according to the general procedure that was used for compounds **18a–g**.

4.3.1. 2(*S*)-*tert*-Butyl 2-isopropyl-3-oxo-1,2,3,4,5,10-hexahydroazepino[3,4-*b*]indol-4-yl carbamate 18h. Yield: 22%; $[\alpha]_D^{20}$ +143.2 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3315, 2977, 1689, 1631, 1164, 738; HPLC (grad. 1): t_R=23.40 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.62; MS: 358 $[M+H]^+$, 380 $[M+Na]^+$, 737 $[2M+Na]^+$; ^1H NMR (200 MHz, $CDCl_3$): δ 0.94 (d, *J*=6.8 Hz, 3H), 1.16 (d, *J*=6.8 Hz, 3H), 1.49 (s, 9H), 2.81 (pseudo-t, 1H), 3.27 (pseudo-d, 1H), 4.08 (d, *J*=17.4 Hz, 1H), 4.78 (d, *J*=17.4 Hz, 1H), 4.87–5.11 (m, 2H), 6.05 (d, *J*=7.2 Hz, 1H), 7.01–7.36 (m, 4H), 8.22 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.9 (CH_3), 20.2 (CH_3), 28.4 (CH_3), 29.1 (CH_2), 38.1 (CH_2), 45.2 (CH), 50.9 (CH), 79.7 (C), 109.7–134.7 (C+CH), 155.2 (C), 171.8 (C); HRMS (ESI^+) exact mass calculated for $C_{20}H_{27}N_3O_3Na$ $[M+Na]^+$: 380.1950, found: 380.1945.

4.3.2. 2(*S*)-*tert*-Butyl 2-isobutyl-3-oxo-1,2,3,4,5,10-hexahydroazepino[3,4-*b*]indol-4-yl carbamate 18i. Yield: 38%; $[\alpha]_D^{20}$ +136.8 (*c* 1, $CHCl_3$); IR (neat, cm^{-1}): 3313, 2964, 1691, 1638, 1155, 736; HPLC (grad. 1): t_R=24.72 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.66; MS: 394 $[M+Na]^+$, 765 $[2M+Na]^+$; ^1H NMR (200 MHz, $CDCl_3$): δ 0.73 (d, *J*=6.6 Hz, 3H), 0.84 (d, *J*=6.6 Hz, 3H), 1.49 (s, 9H), 1.72–1.93 (m, 1H), 2.80 (pseudo-t, 1H), 3.14–3.48 (m, 2H), 3.95 (d, *J*=17.0 Hz, 1H), 4.98–5.06 (m, *J*=16.6 Hz, 2H), 6.03

(d, J=7.2 Hz, 1H), 7.00–7.33 (m, 4H), 8.37 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.8 (CH_3), 20.0 (CH_3), 27.6, 28.4 (CH_3), 29.0 (CH_2), 45.8 (CH_2), 50.9 (CH), 56.3 (CH_2), 79.7 (C), 109.5–134.7 (C+CH), 155.3 (C), 172.5 (C); HRMS (ESI$^+$) exact mass calculated for $C_{21}H_{29}N_3O_3Na$ $[M+Na]^+$: 394.2107, found: 394.2102.

4.3.3. 2(*S*)-*tert*-Butyl 2-butyl-3-oxo-1,2,3,4,5,10-hexahydroazepino[3,4-*b*]indol-4-yl carbamate 18j. Yield: 28%; $[\alpha]_D^{20}$ +131.1 (c 1, $CHCl_3$); IR (neat, cm^{-1}): 3315, 2960, 1690, 1636, 1155, 736; HPLC (grad. 1): t_R=24.79 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.65; MS: 294 $[M-Boc+Na]^+$, 394 $[M+Na]^+$; ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.83 (t, J=7.2 Hz, 3H), 1.15–1.42 (m, 4H), 1.49 (s, 9H), 2.78 (pseudo-t, 1H), 3.24 (pseudo-d, 1H), 3.32–3.58 (m, 2H), 3.96 (d, J=17.2 Hz, 1H), 4.98–5.06 (m, J=17.0 Hz, 2H), 6.02 (d, J=7.0 Hz, 1H), 7.00–7.33 (m, 4H), 8.34 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.9 (CH_3), 27.1 (CH_2), 28.4 (CH_3), 29.0 (CH_2), 30.1 (CH_2), 45.2 (CH_2), 48.7 (CH_2), 50.9 (CH), 79.7 (C), 109.5–134.7 (C+CH), 155.3 (C), 172.2 (C); HRMS (ESI$^+$) exact mass calculated for $C_{21}H_{29}N_3O_3Na$ $[M+Na]^+$: 394.2107, found: 394.2115.

4.3.4. 2(*S*)-*tert*-Butyl 2-cyclohexyl-3-oxo-1,2,3,4,5,10-hexahydroazepino[3,4-*b*]indol-4-yl carbamate 18k. Yield: 24%; $[\alpha]_D^{20}$ +88.3 (c 1, $CHCl_3$); IR (neat, cm^{-1}): 3289, 2930, 1698, 1621, 1698, 1620, 1154, 735; HPLC (grad. 1): t_R=25.99 min; R_f (CH_2Cl_2–EtOAc 1:1): 0.75; MS 320 $[M-Boc+Na]^+$, 420 $[M+Na]^+$; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.19–1.23 (m, 10H), 1.49 (s, 9H), 2.82 (pseudo-t, 1H), 3.26 (pseudo-d, 1H), 4.15 (d, J=17.2 Hz, 1H), 4.52 (m, 1H), 4.82 (d, J=16.8 Hz, 1H), 5.02–5.14 (m, 1H), 6.09 (d, J=7.6 Hz, 1H), 7.00–7.36 (m, 4H), 8.25 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 24.8–30.8 (CH_2), 28.4 (CH_3), 29.2 (CH_2), 39.1 (CH_2), 50.9 (CH), 53.5 (CH), 79.9 (C), 109.7–134.7 (C+CH), 155.3 (C), 172.1 (C); HRMS (ESI$^+$) exact mass calculated for $C_{23}H_{31}N_3O_3Na$ $[M+Na]^+$: 420.2263, found: 420.2249.

4.4. Boc-Tyr-Pro-Aia-Phe-OMe 19

Boc-Aia-Phe-OMe **18g** (0.150, 0.31 mmol) was treated with 1 M HCl–EtOAc (2 mL). After 3 h, cold Et_2O was added to precipitate the product. Aia-Phe-OMe·HCl (0.119 g, 0.288 mmol) was suspended in CH_2Cl_2 (6 mL). Diisopropylethylamine (0.126 mL, 0.72 mmol), Boc-Tyr-Pro-OH (0.109 g, 0.288 mmol), HOBt (0.039 g, 0.288 mmol), and TBTU (0.102 g, 0.317 mmol) were added. The reaction mixture was stirred at 0 °C for 1 and 3 h at room temperature until TLC indicated the absence of the substrates. The solvent was evaporated and the residue was redissolved in EtOAc (15 mL) and washed with 1 M HCl (3×6 mL), saturated $NaHCO_3$ (3×6 mL), and brine (3×6 mL). The organic layer was dried over $MgSO_4$. After evaporation of the solvent, the crude product was obtained (0.197 g, 93%). HPLC (grad. 1): t_R=15.5 min; MS: 738 $[M+H]^+$.

4.5. Synthesis and characterization of 2-[4-(acetylamino)-3-oxo-3,4,5,10-tetrahydroazepino[3,4-*b*]indol-2(1*H*)-yl]-3-phenylpropanamide 8

Compound **18g** (0.20 g, 0.42 mmol) was dissolved in a solution of NH_3 in MeOH (25%, 20 mL). The vessel was sealed tightly with a cap and the reaction was stirred during 2 days. Thirty percent of racemization occurred. The solution was evaporated and the crude product was used in the following step. The Boc protecting group was removed as described for compound **18b**. The resulting TFA salt (0.42 mmol) was dissolved in water (4 mL). The pH was adjusted to 6 with NEt_3 and Ac_2O (0.2 mL, 2.1 mmol) was added in three portions. Meanwhile the pH was kept at 6 with NEt_3. After stirring for 2 h at room temperature, HPLC analysis showed completion of the reaction. The reaction mixture was evaporated and the residue was purified by preparative HPLC (gradient 2).

Yield: 43% over three steps; $[\alpha]_D^{20}$ −17.2 (c 1.25, MeOH); IR (neat, cm^{-1}): 3328, 2978, 3361, 2330, 1638, 1182; HPLC (grad. 2): t_R=11.39 min; R_f (EtOAc): 0.16; MS 404 $[M]^+$; ^{1}H NMR (500 MHz, DMSO): δ 10.82 (s, 1H), 8.09 (d, J=6.5 Hz, 1H), 7.31–6.82 (m, 11H), 5.14 (t, J=6.9 Hz, 1H), 5.07–5.05 (m, 1H), 4.94 (d, J=16.8 Hz, 1H), 4.59 (d, J=16.8 Hz, 1H), 3.27–3.23 (m, 1H), 3.00 (d, J=14.0 Hz, 1H), 2.95–2.92 (m, 1H), 2.7 (t, J=14.0 Hz, 1H), 1.92 (s, 3H); ^{13}C NMR (126 MHz, DMSO): δ 172.1 (C), 171.0 (C), 168.7 (C), 137.7–106.8 (C+CH), 58.9 (CH), 49.9 (CH), 41.2 (CH_2), 34.7 (CH_2), 27.5 (CH_2), 22.6 (CH_3); HRMS (ESI$^+$) exact mass calculated for $C_{23}H_{24}N_4O_3$ $[M+H]^+$: 405.1927, found: 405.1868.

4.6. Synthesis of Tyr-Pro-Aia-Phe-NH$_2$ 9

Boc-Tyr-Pro-Aia-Phe-OMe **19** (0.1 g, 0.136 mmol) was dissolved in MeOH saturated with ammonia (5 mL). After 72 h, the solvent was evaporated to give a yellow solid. The peptide amide was treated with 1 M HCl–EtOAc (2 mL). After 2 h, cold Et_2O was added to precipitate the product. Analytical HPLC indicated two epimeric forms of the peptide (ratio 66:34). Semi-preparative HPLC yielded 31% of **9** (purity>99%). HPLC (grad. 2): t_R=14.23 min; MS: 606 $[M-NH_2]^+$, 623 $[M+H]^+$, 645 $[M+Na]^+$.

4.7. Synthesis of Tyr-Pro-Aia-Phe-OH 21

To a solution of Boc-Tyr-Pro-Aia-Phe-OMe **19** (0.0858 g, 0.116 mmol) in MeOH (1.5 mL) at 0 °C, LiOH (0.58 M, 1 mL) was added and the reaction mixture was stirred for 5 h at this temperature. MeOH was evaporated, water was added (2 mL) and acidified to pH 3.0 with 10% citric acid. The aqueous phase was extracted with EtOAc (4×3 mL), combined organic layers were washed with brine (3×2 mL), and dried over $MgSO_4$ to give a yellow foam after evaporation. The peptide acid was treated with 1 M HCl–EtOAc (2 mL). After 2 h, cold Et_2O was added to precipitate the product. Semi-preparative HPLC yielded 44% of **21** (purity>99%). HPLC (gradient 2): t_R=14.60 min; MS: 624 $[M+H]^+$, 646 $[M+Na]^+$.

4.8. Procedure for the Boc-deprotection of 18c and its derivatization with Marfey's reagent (FDAA)

Analog **18c** (0.013 g, 0.028 mmol) was dissolved in a mixture of TFA–water 95:5 (231 μL) and CH_2Cl_2 (100 μL) was added. The mixture was stirred for 1 h at room temperature. The solution was evaporated to give the crude TFA-salt.

Table 4. The 10 lowest-energy conformers of Ac-Aia-Phe-NH_2 **8** after clustering into families

Conformer number	Potential energy (kJ/mol)	Relative energy (kJ/mol)	φ_2 (°)	ψ_2 (°)	φ_3 (°)	ψ_3 (°)	χ^1 (Trp, °)	χ^1 (Phe, °)
1	118.21	0.00	−161	159	68	19	−175	−53
2	118.72	0.51	−161	158	−123	37	−174	−53
3	126.50	8.29	−67	−52	−121	33	60	−52
4	127.76	9.55	−162	160	76	123	−177	−55
5	130.51	12.30	−161	161	−134	38	−172	55
6	131.30	13.09	180	−59	−125	37	58	−53
7	132.11	13.90	−161	160	−119	107	−175	−173
8	133.77	15.56	85	153	−123	35	−166	−53
9	134.06	15.85	85	153	67	20	−167	−53
10	134.50	16.29	−161	159	−125	−107	−175	−59

A stock solution of the TFA-salt of the analyte (0.005 g, 0.009 mmol) in acetone–water 1:1 (1 mL) was prepared and triethylamine was added to this solution (0.13 μL).

A stock solution of FDAA (0.005 g, 0.183 mmol) in acetone (1 mL) was prepared.

FDAA-solution (40 μL, 0.007 mmol) was added to the analyte stock solution (28 μL, 0.0003 mmol) and the mixture was incubated at 40 °C overnight. The mixture was then quenched with aqueous HCl (1M, 28 μL) and the solution was diluted to 1 mL with acetonitrile–water 1:1.

HPLC analysis was performed using gradient 1 (λ=340 nm). Retention times were 24.01 min (*S*) and 25.35 min (*R*).

4.9. Molecular modeling of Ac-Aia-Phe-NH_2 8

The calculations were carried out using MacroModel 5.0[39] with Maestro 8.0 as a graphic interface. The MM3* force field[40] was used for energy minimization in combination with the GB/SA solvation model of Still et al.,[41] using MacroModel's default parameters for an aqueous medium. The conformational analysis of Ac-Aia-Phe-NH_2 **8** structure was carried out with the Pure Low Mode search.[42] Structures (130,000) were generated and minimized by means of the Polak–Ribière conjugate gradient method as implemented in MacroModel, using a gradient convergence criterion of 0.1 kJ/mol Å. After this search the found conformations were again minimized to an energy convergence of 0.01 kJ/mol Å. Duplicate structures and those greater than 50 kJ/mol above the global minimum were discarded. The generated structures were clustered in families with Xcluster 1.7. A RMSD value of 0.2 Å was used.

This revealed 10 low energy conformations of Ac-Aia-Phe-NH_2 **8** in a range of 16.74 kJ/mol above the global minimum (E=118.21 kJ/mol) (Table 4).

4.10. Superimposition of Ac-Aia-Phe-NH_2 8 with the C-terminus of the *trans*-endomorphin-1 NMR structure

Ac-Trp-Phe-NH_2 has been built using the torsional angles of the *trans*-endomorphin-1 NMR structure:[25] $(\varphi,\psi)_{i+1}$=(−106, −179), $(\varphi,\psi)_{i+2}$=(−50,112) and with χ^1 (Trp)=−39° and χ^1 (Phe)=−179°. The MM3* force field[40] was used in vacuo for the energy minimization of this structure. The minimization with constraints was carried out with the Polak–Ribière conjugate gradient method as implemented in MacroModel, using a gradient convergence criterion of 0.02 kJ/mol Å.

The superimposition of all the backbone atoms of Ac-Trp-Phe-NH_2 with those of conformer 7 (Table 3) was carried out with MacroModel 5.0.[39]

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