JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Labelled Cpd Radiopharm 2001; **44**: 501–508.
DOI: 10.1002/jlcr.477

Synthesis of tritium labelled delta sleep-inducing peptide

Matthieu Giraud[1], Jean-Louis Morgat[2], Florine Cavelier[1,*] and Jean Martinez[1]

[1] *Laboratoire des Aminoacides, Peptides et Protéines, CNRS-UMR 5810, Universités Montpellier I et II CC19, 34095 Montpellier Cedex 5, France*
[2] *Centre de Recherche sur les Biopolymères Artificiels, ESA-CNRS 5473, Faculté de Pharmacie, 15 Av. Ch. Flahault, 34060 Montpellier Cedex 2, France*

Summary

The α,β-dehydro precursor of the delta sleep-inducing peptide (DSIP) for tritiation was prepared prior to tritiation using a 3 + 6 fragment coupling strategy on a solid support. The first fragment, α,β-dehydrotripeptide, was prepared in solution in five steps in 79% overall yield while the second fragment was obtained by a step by step peptide synthesis on a Wang resin using an Fmoc strategy. The α,β-dehydrotripeptide was coupled to the fragment linked to the resin, followed by a deprotection/cleavage step to yield the α,β-dehydro-DSIP, **7**. Catalytic reduction of unsaturated DSIP using tritium gas and palladium oxide as catalyst gave [^{3}H]DSIP having a specific activity of 1.184 TBq/mmol(32 Ci/mmol). Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: delta sleep-inducing peptide (DSIP); tritium labelling; dehydropeptide; opioid; alcohol withdrawal

1. Introduction

Delta sleep-inducing peptide (DSIP) is a nonapeptide first isolated from cerebral blood dialysate of the rabbit. It has been shown to possess delta-EEG enhancing activity.[1] It exhibits a wide range of modulatory

*Correspondence to: F. Cavelier, LAPP, CNRS-UMR 5810, UM II CC19, 34095 Montpellier Cedex 5, France.

Received 22 January 2001
Revised 8 March 2001
Accepted 12 March 2001
Published online ▮▮▮▮

effects, it has an effect on circadian rhythms by interacting with serotonin and melatonin,[2,3] on temperature regulation[4–6] and on resistance against acute emotional stress.[7] It is also connected with inhibition of histamine and activation of GABA-ergic systems[8,9] and with inducing cerebral monoamine oxidase activity.[10–12] Finally, it has been postulated that DSIP modulates endogenous opioid–peptidergic systems and exogenous intracerebrally or systemically administered morphine.[13–16] Unfortunately, its mechanisms of action have never been fully determined. The objective of looking for a binding site for DSIP prompted us to investigate the synthesis of [^{3}H]DSIP.

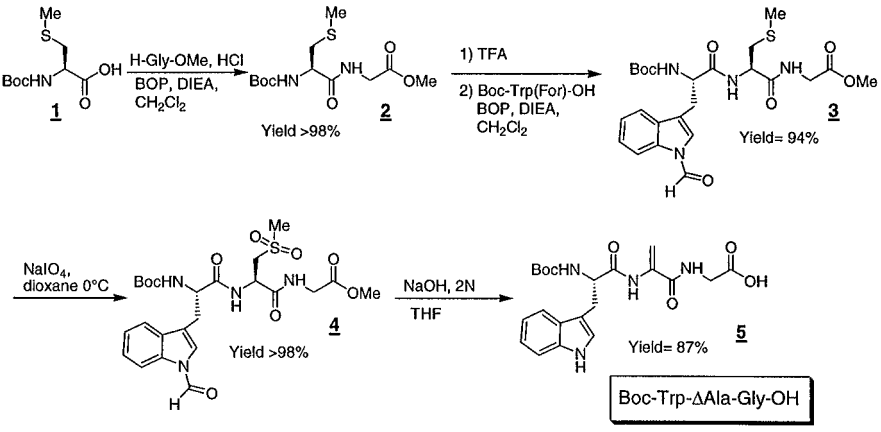
Results and discussion

The catalytic reduction of a double bound is a suitable reaction to obtain tritium labelled compounds with high specific activity. The DSIP (H–Trp–Ala–Gly–Gly–Asp–Ala–Ser–Gly–Glu–OH) has in position 2 an alanine residue. We introduced an unsaturated precursor, the α,β-dehydroalanine (ΔAla), in its place in the peptide sequence. For the peptide synthesis, the glycine in position 3 allowed us to consider a 3 + 6 fragment coupling strategy without any racemization risk.

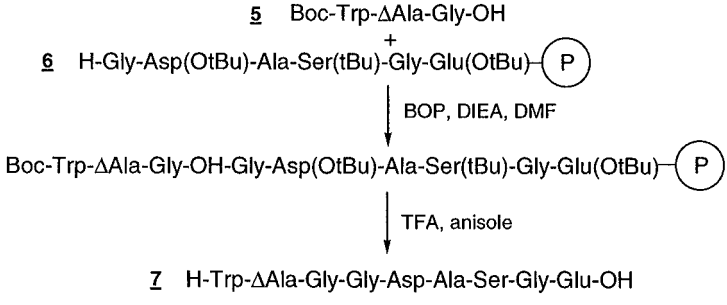
The synthesis of the tripeptide fragment possessing the α,β-dehydroamino acid was carried out in solution with a Boc strategy and BOP[17] as a coupling reagent. The glycine methyl ester hydrochloride (**1**) was coupled to Boc–Cys(Me)–OH in quantitative yield. The dipeptide (**2**) was treated with trifluoroacetic acid and the resulting trifluoroacetate salt was coupled with Boc–Trp(For)–OH under the same coupling conditions to yield the tripeptide (**3**). The formyl group was used to prevent tryptophan oxidation during the next step. The cysteine sulphur was then oxidized with sodium periodate in dioxane to yield the compound (**4**). Finally, trytophan deprotection, methyl ester saponification and β-elimination of tripeptide (**4**) were performed in a single step using an alkaline treatment (aqueous 2 N NaOH) in THF to yield the desired α,β-dehydrotripeptide (**5**) (Scheme 1).

The second fragment (**6**) was synthesized on a pre-loaded Fmoc–Glu(OtBu)–Wang resin (0.56 mmol/g) with standard Fmoc chemistry[18] (see experimental part). The two fragments were coupled on resin with BOP (Scheme 2).

Cleavage from the support and deprotection with trifluoroacetic acid containing appropriate scavengers afforded the crude peptide,



Scheme 1. α,β**-dehydrotripeptide synthesis**



Scheme 2. α,β**-dehydro-DSIP fragment coupling strategy**

which was purified by preparative HPLC to yield the ΔDSIP (7) (yield 45%).

The tritiation of ΔDSIP with tritium gas (0.5 bar) was performed in dioxane/water (50/50 v:v) for 1 h at room temperature in the presence of PdO as a catalyst. Hydrochloride acid (1 N) was added to the reaction mixture to avoid peptide complexation with the catalyst. The two DSIP analogues containing L-alanine and D-alanine in position 2 displayed distinct retention time (3 min difference) in the HPLC (Figure 1) and were easily separated. Identification of the L-Ala containing isomer was performed by comparison with natural DSIP co-injection. The specific activity of tritium labelled [^{3}H]DSIP was 1.184 TBq/mmol (32 Ci/mmol).

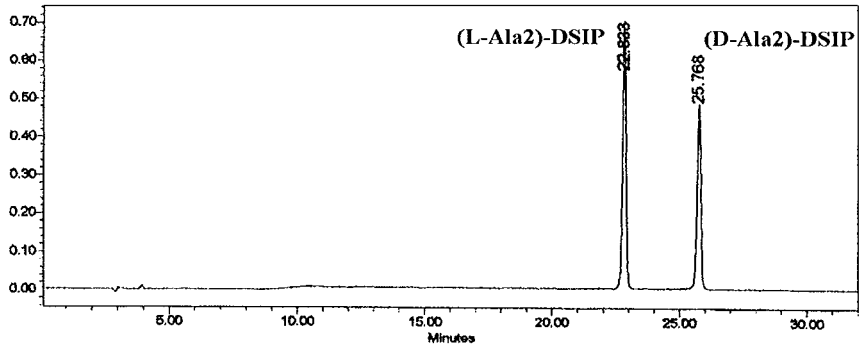


Figure 1. HPLC profile of the catalytic reduction chromatogram of ΔDSIP

Experimental

Amino acid derivatives were purchased from Senn Chemicals, pre-loaded resins from Novabiochem, anisole from Prolabo and triisopropylsilane from Fluka. TFA of synthesis quality, was purchased from Merck and distilled. Dichloromethane was dried overnight on $CaCl_2$, then distilled from K_2CO_3 and stored away from light in a brown bottle. Diisopropylethylamine was distilled over KOH/ninhydrin and stored away from light. Water was obtained from Milli-Q plus system (Millipore), acetonitrile from Merck.

Thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60F254 plates and spots were visualized by ultraviolet light or by staining with phosphomolybdic acid. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). The HPLC analyses were carried out on Waters Millenium with a photodiode Array detector 996, wavelength 214 nm, using a reversed phase Nucleosil C18, 5 μ, 250 × 10 mm and a flow of 1 ml/min, using a 30 min gradient of 10–100% aqueous acetonitrile containing 0.1% TFA, solvent A: H_2O, TFA 0.1%, solvent B: CH_3CN, TFA 0.1%. The preparative apparatus was a Waters PrepLC 4000 system with a reversed phase Nucleosil Delta Pak C_{18}, 15 μ, 100 Å, 40 × 100 mm and a Guard Pak C_{18}, 15 μ, 100 Å, 40 × 10 mm in a PrepLC 40 mm Chamber Assembly. The Tunable Absorbance Detector was a Waters 486 operated at 214 nm.

Tritium gas was supplied by RC Tritec (Switzerland). The specific activity was determined after tritium counting either by measuring the OD or by comparative quantification of the HPLC chromatogram (UV)

detection with a known concentration of a non-labelled DSIP sample. Radioactivity [^{3}H] was determined using a Packard liquid scintillation counter (TRI-Carb 2100 TR). The tritium manifold system was built by RC Tritec LTD (Switzerland).

*Boc–Cys(Me)–Gly–OMe (**2**)*

Diisopropylethylamine (*ca* 15 mmol) was added to Boc–Cys(Me)–OH (**1**) (1.17 g, 5 mmol), HCl, H–Gly–OMe (750 mg, 6 mmol) and BOP (2.65 mg, 3.87 mmol) in dichloromethane (60 ml). The pH was adjusted to 8–9 during the coupling reaction. The solvent was removed under reduced pressure and the residue purified by chromatography on silica gel using ethyl acetate/hexane (2:8) without any work up. The dipeptide (**2**) was obtained as a colourless oil. Yield $>98\%$ (1.5 g); R_f: 0.55 (ethyl acetate); R_f: 0.35 (acetate/hexane; 2:8); ESI + ($C_{12}H_{22}N_2O_5S$): 307 $[M+H]^+$, 329 $[M+Na]^+$, 251 $[M+H\text{-isobutene}]^+$, 207 $[M+H\text{-Boc}]^+$, 613 $[2M+H]^+$, 635 $[2M+Na]^+$; RMN ^{1}H ($CDCl_3$), $\delta = 1.35$ (s, 9H, Boc), 2.05 (s, 3H, SCH_3), 2.8 (d, $J = 6.4$ Hz, 2H, $H_{\beta\beta'}$), 3.65 (s, 3H, OCH_3) 3.95 (m, 2H, $H_{\alpha\alpha'}$), 4.3 (m, 1H, H_α), 5.55 (d, $J = 8$ Hz, 1H, NHCys), 7.15 (t, $J = 5$ Hz, 1H, NHGly); HPLC:$t_R = 18.7$ min(10% A–30 min–$>$100% A).

*Boc–Trp(For)–Cys(Me)–Gly–OMe (**3**)*

TFA (30 ml) was added to Boc–Cys(Me)–Gly–OMe (**2**) and the resulting reaction mixture was stirred at room temperature for 30 min. TFA was removed under reduced pressure. Diisopropylethylamine (*ca* 2.5, eq, pH 8–9) was added to the crude dipeptide trifluoroacetate salt, Boc–Trp(For)–OH (2.6 g, 7.84 mmol) and BOP (3.5 g, 7.84 mmol) in dichloromethane (40 ml). The solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (100 ml) and the organic layer washed successively with 1 N $KHSO_4$ (3×50 ml), brine (50 ml), saturated $NaHCO_3$ (3×50 ml) and brine (50 ml). The organic phase was dried over $MgSO_4$ and concentrated to dryness. The residue was purified by chromatography on silica gel using ethyl acetate/hexane (2:8) as eluent. The tripeptide was obtained as a white solid. Yield: 84% (1.71 g); R_f: 0.50 (ethyl acetate); ESI + ($C_{24}H_{32}N_4O_7S$): 521 $[M+H]^+$, 543 $[M+Na]^+$, 465 $[M+H\text{-isobutene}]^+$; 276 $[(M+H+Na)/2]^{++}$, RMN ^{1}H ($CDCl_3$), $\delta =$ 1.40 (s, 9H, Boc), 2.10 (s, 3H, SCH_3), 2.8 (m, 2H, $H_{\beta\beta'}$Trp), 3.3 (d,

$J = 6.4$ Hz, 2H, $H_{\beta\beta'}$Cys), 3.75 (s, 3H, OCH_3), 4.0 (m, 2H, $H_{\alpha\alpha'}$), 4.6 (m, 2H, H_αCys + H_αTrp), 5.3 (m, 1H, NHTrp), 7.1 (m, 2H, NHCys + NHTrp), 7.5 (m, 5H, Ar), 9.1(s, 1H HFor), HPLC: $t_R = 23.21$ min (10% A–30 min– > 100% A).

*Boc–Trp(For)–Cys(OMe)–Gly-OMe (**4**)*

A solution of $NaIO_4$ (1 g, 1.94 mmol) in distilled water (8 ml) was added dropwise to the tripeptide Boc–Trp(For)–Cys(Me)–Gly–OMe (**3**) in dioxane (40 ml) at 0° over a period of 30 min. After 1 h, the reaction medium was raised to room temperature and stirred for 16 h. The solvent was removed until *ca* 5 ml and distilled water (10 ml) was added. The aqueous phase was extracted (3×20 ml) with ethyl acetate. The combined organic phases were concentrated to dryness and the crude oxidized compound was used without any purification in the next step. Yield > 98% (1.04 g); R_f: 0.4 (ethyl acetate); ESI + ($C_{24}H_{32}N_4O_8S$): 537 $[M + H]^+$, 559 $[M + Na]^+$, 575 $[M + K]^+$, HPLC: $t_R = 19.05$ min (10% A–30 min– > 100% A).

*Boc–Trp–ΔAla–Gly–OH (**5**)*

2 N NaOH (3.75 ml, 7.5 mmol) was added dropwise to the tripeptide Boc–Trp(For)–Cys(OMe)–Gly–OMe (**4**) (800 mg, 1.5 mmol) in THF (10 ml) at 0°C over a period of 30 min. After 1 h stirring at room temperature, the solvent was removed under reduced pressure and the residue was dissolved in distilled water (10 ml). The aqueous phase was washed twice with ether. The pH was raised to 4–5 with 1 N $NaHSO_4$ and the resulting aqueous solution was extracted with ethyl acetate (3×40 ml). The organic phases were combined, dried over magnesium sulphate and concentrated under reduced pressure. The dehydrotripeptide was obtained as a white solid. Yield: 87% (561 mg); R_f: 0.2 ($CHCl_3$/MeOH/AcOH, 85:10:5); ESI + ($C_{21}H_{26}N_4O_6$): 431 $[M + H]^+$, 453 $[M + Na]^+$, 861 $[2M + H]^+$, 883 $[2M + Na]^+$, RMN ^1H ($CDCl_3$), $\delta =$ 1.35 (s, 9H, Boc), 2.8 (m, 2H, $H_{\beta\beta'}$Trp), 4.0 (m, 2H, $H_{\alpha\alpha'}$), 4.3 (m, 1H, H_αTrp), 5.2 (m, 2H, H_αΔAla + NHTrp), 5.65 (d, 1H, J = 7.5 Hz, H_βΔAla), 7.5 (m, 6H, Ar + NHGly), 9.3 (s, 1H, NHΔAla), HPLC: $t_R = 18.70$ min (20% A–30 min– > 50% A).

*Peptide synthesis on Wang resin H–Gly–Asp(OtBu)–Ala–Ser(tBu)–Gly–Glu(OtBu)–● (**6**)*. The hexapeptide was synthesized using standard Fmoc chemistry[19] first introduced by Carpino and

Han,[18,20] on a 433A peptide synthesizer with HBTU/HOBt-activation. Fmoc amino acids were coupled with the BOP reagent and the reaction was monitored with the colour Kaiser test.[21] Deprotection steps were carried out with piperidine (20%) in DMF.

Fragment peptide coupling (H–Trp–ΔAla–Gly–Gly–Asp–Ala–Ser–Gly–Glu–OH) (7): Diisopropylethylamine (*ca* 2 mmol, pH 8–9) was added to Boc–Trp–ΔAla–Gly–OH (**5**) (430 mg, 1 mmol), BOP (442 mg, 1 mmol) and the hexapeptide (0.25 mmol) linked to the resin suspended in DMF in a suitable peptide vessel for manual peptide synthesis. After overnight stirring, the solvent was filtered off and washed with DMF (3 times). A negative Kaiser test was obtained. TFA (25 ml/g resin) containing appropriate scavengers (anisole, triisopropylsilane) was added to the dry resin. After 2 h of stirring, the resin was filtered, followed by a TFA washing. Filtrates were combined and concentrated under reduced pressure. The residue was dissolved in water and the aqueous layer washed with ether (3 times) and then freeze-dried. The crude dehydro-DSIP was purified by a preparative HPLC (0% A–30 min–> 30% A). Solvents were: A, water with 0.1% TFA; B, acetonitrile with 0.1% TFA). Yield: 45%(95 mg); ESI + ($C_{35}H_{48}N_{10}O_{15}$): 847 $[M+H]^+$, 869 $[M+Na]^+$ HPLC: $t_R = 10.2$ min (10% A–30 min–> 100% A).

[^{3}H]delta-sleep inducing peptide ([^{3}H]DSIP)

Dehydro-DSIP (**7**) (2.3 mg, 2.43 μmol) was solubilized in a water/dioxane solution (1 ml; 50/50 v:v). The reaction medium was frozen in liquid nitrogen and PdO (11.5 mg) was dispersed on the surface and the reacting vial was connected to the micro gas transfer unit. When the vial and all tubing had been evacuated (5.10–3 Torr), pure tritium gas, stored in an activated tritium-U-bed system, was introduced until 0.5 bar and the catalyst was then flushed for 30 min (pressure red at low temperature: −196°C) into the still-frozen solution. After thawing the reaction mixture was magnetically stirred (vigorously) at room temperature for 1 h. The absorption of tritium gas produced a weak pressure reduction. To this mixture 1 N HCl (30 μl) was then added and the catalyst was removed by centrifugation (5000 rpm) and labile tritium atoms were eliminated by two successive flash evaporations. The purity of crude [^{3}H]DSIP was then checked by HPLC (see conditions in materials and methods). The ultra-violet spectrum of the tritiated

peptide was found to be exactly the same as that of the reference. Quantitative and comparative estimation (by UV spectrometric and HPLC titration) indicated and confirmed that the specific activity was 1.184 TBq/mmol (32 Ci/mmol). An overall radiochemical purity of greater than 97% was obtained by HPLC and no significant difference was found in binding studies between the tritiated and non-tritiated product. After eight months of storage at -40°C (65 mM, H_2O/ethanol, 95:5), the tritiated compound retained both their chemical and biological potencies.

References

1. Schoenenberger GA, Monnier M. *Proc. Natl Acad Sci USA* 1986; **74**: 1282–1986.
2. Ouichou A, Pevet P. *Biol Signals* 1992; **1**: 78–87.
3. Yehuda S, Mostofsky, DI. *Peptides* 1993; **14**: 203–205.
4. Tsunashima K, Kato N, Masui A, *et al. Peptides* 1994; **15**: 61–65.
5. Graf MV, Kastin AJ, Coy DH, *et al. Physicol Behav* 1984; **33**: 291–295.
6. Yehuda S, Kastin AJ, Coy DH. *Pharmacol Biochem Behav* 1980; **13**: 895–900.
7. Sudakov KV, Coghlan JP, Kotov AV, *et al. Ann NY Acad Sci* 1995; **771**: 240–251.
8. Shandra AA, Godlevskii LS, Brusentsov AI, *et al. Neurosci Behav Physiol* 1998; **28**: 521–526.
9. Lysenko AV, Alperovich DV, Uskova NI, *et al. Biochemistry (Mosc)* 1999; **64**: 652–657.
10. Dovedova EL, Ashmarin IP. *Biul Eksp Biol Med* 1982; **93**: 56–8.
11. Khvatova EM, Rubanova NA, Prudchenko IA, *et al. FEBS Lett* **368**: 367–369.
12. Voitenko NN, Kolpakov VG, Alekhina TA. *Biul Eksp Biol Med* 1990; **110**: 306–308.
13. Backmund M, Meyer K, Rothenhaeusler HB, *et al. J Clin Psychopharmacol* 1998; **18**: 257–258.
14. Ouichou A, Zitouni M, Raynaud F, *et al. Biol Signals* 1992; **1**: 65–77.
15. Tissot R. *Neuropsychobiology* 1981; **7**: 321–325.
16. Dick P, Grandjean ME, Tissot R. *Neuropsychobiology* 1983; **10**: 205–208.
17. Castro B, Dormoy JR, Evin G, *et al. Tetrahedron Lett* 1975; **14**: 1219–1222.
18. Carpino LA, Han GY. *J Am Chem Soc* 1970; **92**: 5748–5749.
19. Field GB, Noble RL. *Int J Pept Protein Res* 1990; **35**: 161–214.
20. Carpino LA, Han GY. *J Org Chem* 1972; **37**: 3404–3409.
21. Sarin VK, Kent SBH, Tam JP, *et al. Analyt Biochem* 1981; **117**: 147–157.

