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Research Article

Synthesis of doubly ^{13}C-labelled antalarmin isotopomers for pharmacokinetic studies

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Summary

Antalarmin (butyl-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine) was doubly labelled with carbon-13. The

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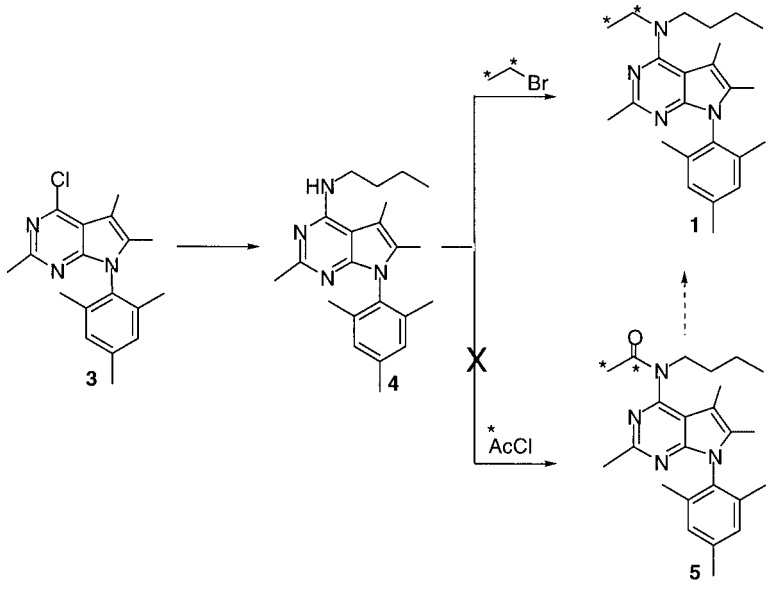
synthesized butyl-[$^{13}C_2$]ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine (**1**) and butyl-ethyl-[2-^{13}C]-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*-pyrrolo[2,3-*d*]-[2-^{13}C] pyrimidin-4-yl]-amine, (**2**) were prepared for use as substrates for pharmacokinetic studies. These compounds were obtained in fair overall yield in a 5 and 6 step synthesis (20–24.5%, respectively) and high isotopic purity (about 99 at% ^{13}C).

Key Words: corticotropin-releasing hormone; CRHR1 antagonist; antalarmin; carbon-13 labelling

1. Introduction

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide, which acts as a major regulator of the hypothalamic–pituitary–adrenal (HPA) axis and is a principal coordinator of stress response including its neuroendocrine, autonomic, immune, and behavioral components.[1–3] In the central nervous system, CRH activates the sympathetic nervous system like a neurotransmitter, thus modulating cardiovascular, metabolic and stress-like behavioral effects associated with drug withdrawal syndromes.[4–9] Overproduction of CRH in the brain has been connected with mental disorders such as anxiety, addiction, and depression, for that reason CRH-antagonists are expected to become a valuable new class of antidepressants.[10–13] Competitive binding studies with ^{125}I-CRH have established that antalarmin and some of its analogues display high affinity and are specific for type 1 CRH receptors. In addition, antalarmin has been shown to block the *in vivo* and *in vitro* biological actions of an CRHR1 agonist.[14]

^{13}C-labelled isotopes have been used for the *in vivo* pharmacokinetic evaluation of drug absorption and disposition.[15,16] Stable isotopes incorporating double ^{13}C-labelling of the highly selective CRHR1 antagonist, antalarmin, were synthesized to explore the pharmacokinetic properties of this pharmacologically important compound. The pharmacokinetic investigation of antalarmin would be helpful in several ways: to determine the pharmacokinetics of the labelled compound in the presence of endogenous antalarmin, to facilitate identification of antalarmin metabolites, and to provide information about a preferred route of administration. Regarding the latter, stable isotope labelled compounds are particularly well suited for pharmacokinetic studies in which a labelled isotopomer and the corresponding non-labelled

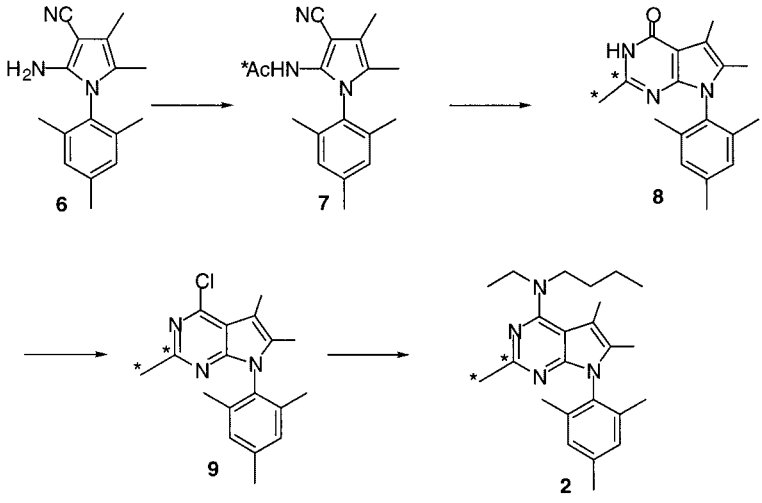


Scheme 1.

compound are administered simultaneously by different routes, e.g., intrathecal and i.v. This type of experiment allows examination of rate and extent of compound transfer between the two sites, in this example between spinal fluid and plasma. Doubly labelled compounds are more useful for pharmacokinetic studies than singly labelled compounds in that they provide better mass spectral separation of the labelled compound from the non-labelled compound. In order to investigate the pharmacokinetics of antalarmin, two labelled derivatives of antalarmin were prepared, and each of these derivatives had their two ^{13}C atoms in different molecular positions that might be subject to differential metabolic cleavage. These labels were placed in the molecular positions seen in **1** (Scheme 1) and **2** (Scheme 2) because the synthetic route to those compounds appeared to be the most facile and likely to be achieved using available starting materials.

Results and discussion

Isotopomer **1** was synthesized as outlined in Scheme 1. The coupling reaction of **3**[17] with *n*-butylamine was carried out according to Chorvat *et al.*[18] and afforded compound **4** in 63% yield.



Scheme 2.

The introduction of the doubly labelled *N*-ethyl group was achieved in one step by alkylation of the secondary amine with $^{13}C_2$-labelled ethyl bromide to give compound **1**. Since $^{13}C_2$-acetyl chloride was less expensive than $^{13}C_2$-labelled ethyl bromide, we initially planned to install the *N*-ethyl group by acetylation of **4** with $^{13}C_2$-acetyl chloride followed by reduction. However, only very low yields of desired product were obtained from this acetylation reaction. In an effort to use only a small excess of $^{13}C_2$-acetyl chloride, the reaction never went to completion.

In compound **2**, two adjacent ^{13}C atoms were incorporated into the pyrrolo-pyrimidine backbone of the antalarmin molecule by the route shown in Scheme 2.

Aminopyrrole **6** was synthesized in one step from 2,4,6-trimethylaniline essentially according to a known reaction sequence.[19] The critical acetylation of **6** was attempted under various conditions, employing different solvents, bases, and both acetyl chloride and acetic anhydride.[19] The best results were obtained using chloroform as a solvent, pyridine as a base and acetyl chloride as alkylating agent. The doubly ^{13}C-labelled compound **7** was obtained in high yield (95%). Cyclization of **7** was carried out in 85% phosphoric acid to afford 4-oxopyrrolopyrimidine derivative **8**. Subsequent reaction with phosphorous oxychloride produced compound **9**, which in turn was coupled with ethyl-*n*-butylamine to give the double ^{13}C-labelled antalarmin

isotopomer **2**. The identity of the two different isotopomers (**1** and **2**) was unequivocally established by the methodology used in their chemical synthesis, and NMR. The chemical shifts of the methyl group on the pyrrolopyrimidine ring in **2**, and the *N*-ethyl moiety in isotopomer **1**, were identical to the comparable atoms in antalarmin. Further, the coupling pattern between the ^{13}C atoms and an attached proton proved the contiguity of the two ^{13}C atoms in both isotopomers. Mass spectral data indicated that isotopomers **1** and **2** were about 99 at% isotopically pure by examination of the M^+-2 peak at *m*/*z* 378 (^{12}C peak). The data were acquired by averaging three electric field scans over a 40 Da range. Triplicate determinations were made for each compound. The peak intensity at *m*/*z* 378 for the labelled compound was about 0.33 times the peak intensity of a deliberately added sample with an additional 2% of the ^{12}C analog (which should, theoretically, now contain 3 at% ^{12}C). Also, it was found to have about 0.2 times the peak intensity of a deliberately added sample with an additional 4% of the ^{12}C analogue (which theoretically should have contained about 5 at% of the ^{12}C compound). Thus, we determined that the synthesized material was of high isotopic purity, *ca*. 99 at% ^{13}C.

Experimental

TLC analyses were carried out on Analtech silica gel GHLF 0.25 mm plates with UV light and I_2 detection. Melting points were measured in open glass capillaries on a Thomas–Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz on a Varian Gemini spectrometer and chemical shifts are reported in ppm relative to tetramethylsilane (TMS). The mass spectra for the determination of the purity of **1** and **2** were obtained with a JEOL SX-102 MS utilizing a direct exposure probe in the EI mode, using an ionizing voltage of 70 V and a source temperature of 150°**C** ($\pm$ 15°**C**); all other compounds were examined in the CIMS mode, as noted. Elemental analyses were performed by Atlantic Microlabs Inc., Norcross, GA. Acetyl-$^{13}C_2$ chloride and bromoethane-$^{13}C_2$ were purchased from Aldrich. The purity of both was 99 at% ^{13}C. The MS of the labelled compounds were compared to the spectra of the authentic unlabelled material to confirm their identity. The purity of the labelled antalarmin isotopomers **1** and **2** was also determined by MS.

Butyl-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-amine (**4**)

4-Chloro-2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*- pyrrolo[2,3-d] pyrimidine (**3**) (5.00 g, 15.9 mmol) and butylamine (7.85 ml, 80.0 mmol) were dissolved in DMSO (35 ml) and heated at 135°C under argon, until TLC (eluent: EtOAc/hexane, 2:8) showed no starting material. The mixture was cooled and extracted between H_2O and petroleum ether (PE). The organic layer was washed with 15% citric acid, dried over Na_2SO_4 and evaporated to give 4.62 g of a yellow oil. This was crystallized from PE to yield 3.49 g (62.5%) of colorless crystals: mp 114–116°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.97 (s, 2H), 4.95 (t, $J = 7$ Hz 1H), 3.61 (m, 2H), 2.45 (s, 3H), 2.41 (s, 3H), 2.33 (s, 3H), 1.90 (s, 3H), 1.84 (s, 6H), 1.67 (m, 2H), 1.49 (m, 2H), 1.00 (t, $J = 7.2$ Hz, 3H); CIMS: m/z 351 (MH^+); Anal. ($C_{22}H_{30}N_4$): C, 75.39, H, 8.63, N, 15.98; found: C, 75.16, H, 8.63, N, 15.83.

[$^{13}C_2$]Ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-amine (**1**).

Butyl-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*-pyrrolo[2,3-d]pyrimidin-4-yl]-amine (**4**) (2.63 g, 7.50 mmol) was dissolved in DMSO (52.6 ml) and cooled to 0°C under argon. NaH (0.54 g, 22.5 mmol, 95% dispersion) was added slowly, and the mixture stirred for 20 min. Then double ^{13}C-labelled ethyl bromide (1.00 g, 9.01 mmol) was added and stirring continued at room temperature for 3 h. The mixture was poured into water, and extracted with ethyl acetate (3 × 80 ml). The combined organic layers were washed with brine (3 × 50 ml), dried over Na_2SO_4 and evaporated to give a yellow oil (2.98 g). This was crystallized from MeOH/H_2O to give 2.51 g (88.3%) colorless crystals. mp 80–81°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.97 (s, 2H), 3.59 (m, $^1J_{H,13C} = 145.8$ Hz, $^2J_{H,13C} = 2.7$ Hz, $^3J_{H,H} = 7.2$ Hz, 2H), 3.56–3.50 (m, 2H), 2.46 (s, 3H), 2.37 (s, 3H), 2.34 (s, 3H), 1.93 (s, 3H), 1.84 (s, 6H), 1.65–1.58 (m, 2H), 1.20 (m, $^1J_{H,13C} = 126.0$ Hz, $^3J_{H,H} = 3.9$ Hz, 3H); CIMS: m/z 379 ($M\text{-}1^+$); Anal. ($C_{24}H_{34}N_4$): C, 76.15, H, 9.05, N, 14.08; found: C, 76.07, H, 9.08, N, 14.67.

N-[3-Cyano-4,5-dimethyl-1-(2,4,6-trimethyl-phenyl)-1H-pyrrol-2-yl][$^{13}C_2$]-acetamide (**7**).

2-Amino-4,5-dimethyl-1-(2,4,6-trimethyl-phenyl)-1*H*- pyrrole-3-carbonitrile (**6**) (5.73 g, 22.6 mmol) was dissolved in $CHCl_3$ (14 ml). After

addition of dry pyridine (2 ml), the mixture was cooled under argon atmosphere to 0°C. Doubly ^{13}C-labelled acetyl chloride (2.00 g, 24.9 mmol) was slowly added, while allowing the temperature to rise to 20°C. Stirring at rt was continued until TLC showed no more starting material. The reaction mixture was washed with saturated $NaHCO_3$ (3×50 ml), then 2% HCl (3×30 ml), and H_2O (2×30 ml). The organic layer was dried over Na_2SO_4 and evaporated to give a yellow oil. This oil was crystallized from ether to afford 6.35 g (95%) as colorless crystals, m.p. 121–122°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.96 (s, 2H), 2.33 (s, 4.5H), 2.16 (s, 4.5H), 1.90 (s, 6H), 1.78 (s, 3H), 1.57 (s, 3H); CIMS: *m/z* 297 (MH^+); Anal. ($C_{18}H_{21}N_3O \cdot 0.3H_2O$): calc.: C, 71.87, H, 7.24, N, 13.97; found: C, 71.92, H, 7.06, N, 14.00.

[2-^{13}C]-2,5,6-Trimethyl-7-(2,4,6-trimethyl-phenyl)-3,7-dihydro-pyrrolo[2,3-d]-[2-^{13}C]pyrimidin-4-one (**8**)

N-[3-Cyano-4,5-dimethyl-1-(2,4,6-trimethyl-phenyl)-1*H*- pyrrol-2-yl] [$^{13}C_2$]-acetamide (**7**) (5.60 g, 18.8 mmol), AcOH (7.36 ml) and 85% H_3PO_4 (7.36 ml) were combined and heated at 120°C until TLC (eluent: $CHCl_3$/MeOH/NH_4OH, 90:10:1) showed no more starting material. The mixture was diluted by rapid and dropwise addition of H_2O (14.9 ml) while cooling to 20°C. The mixture was filtered and washed with 33% AcOH (14.9 ml) to afford 4.55 g (82%) of the desired material as colorless crystals: mp >220°C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99 (s, 2H), 2.61 (d, $J = 7.2$ Hz, 1.5H), 2.43 (s, 3H), 2.35 (s, 3H), 2.18 (d, $J = 7.2$ Hz, 1.5H), 1.88 (s, 3H), 1.87 (s, 6H); CIMS: *m/z* 297 (MH^+); Anal. ($C_{18}H_{21}N_3O \cdot 0.25H_2O$): calc.: C, 72.09, H, 7.22, N, 14.01; found: C, 72.21, H, 7.06, N, 14.01.

4-Chloro-[2-^{13}C]-2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-d]-[2-^{13}C]pyrimidine (**9**)

[2-^{13}C]-2,5,6-Trimethyl-7-(2,4,6-trimethyl-phenyl)-3,7-dihydro- pyrrolo [2,3-d]-[2-^{13}C]pyrimidin-4-one (**8**) (4.00 g, 13.5 mmol) and $POCl_3$ (5 ml) were combined under argon atmosphere and heated to reflux until TLC (eluent: $CHCl_3$/MeOH/NH_4OH, 390:90:1) showed no more starting material. The mixture was cautiously poured into H_2O (30 ml), while the temperature was allowed to rise to 60°C. The product was extracted with $CHCl_3$ (80 ml), the combined organic layers were neutralized with saturated $NaHCO_3$. After drying over Na_2SO_4 the solvent was

evaporated to give a colorless foam. Crystallization from EtOH afforded 3.88 g (91.3%) of colorless crystals: mp 204–206°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.02 (s, 2H), 2.82 (d, $J = 7.2$ Hz, 1.5H), 2.47 (s, 3H), 2.39 (d, $J = 7.2$ Hz, 1.5H), 2.36 (s, 3H), 2.00 (s, 3H), 1.82 (s, 6H); CIMS: m/z 315 (MH^+); Anal. ($C_{18}H_{20}N_3Cl \cdot 0.1H_2O$): calc.: C, 68.49, H, 6.45, N, 13.31, Cl, 11.23; found: C, 68.39, H, 6.44, N, 13.36, Cl, 11.39.

Butyl-ethyl-[2-^{13}C]-[2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-d]-[2-^{13}C]pyrimidin-4-yl]-amine (**2**)

4-Chloro-[2-^{13}C]-2,5,6-trimethyl-7-(2,4,6-trimethyl-phenyl)-7*H*-pyrrolo[2,3-d]-[2-^{13}C]pyrimidine (**9**) (1.90 g, 6.02 mmol) and *N*-ethylbutylamine (4.15 ml, 30.4 mmol) were dissolved in DMSO (14 ml) and heated to reflux at 135°C under argon, until TLC (EtOAc/petroleum ether (PE), 1:7) showed no more starting material. The mixture was cooled and treated with 5% NaOH (2 ml) and PE (50 ml) to give two phases. The organic layer was washed with H_2O (3 × 50 ml), dried over Na_2SO_4 and evaporated to give of a yellow oil (2.15 g). This was crystallized from MeOH/H_2O to give 1.83 g (80%) colorless crystals: m.p. 81–85°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.97 (s, 2H), 2.45 (m, 4H), 2.36 (s, 3H), 2.45 (m, $^1J_{H,13C} = 126.6$ Hz, $^2J_{H,13C} = 6.9$ Hz, 3H), 2.34 (s, 3H), 1.93 (s, 3H), 1.84 (s, 6H), 1.62 (m, 2H), 1.36 (s, 2H), 1.20 (t, $J = 6.9$ Hz, 3H), 0.91 (t, $J = 7.2$ Hz, 3H); CIMS: m/z 380 (MH^+); Anal. ($C_{24}H_{34}N_4 \cdot 0.25$ H_2O): calc.: C, 75.25, H, 9.08, N, 14.62; found: C, 75.05, H, 8.81, N, 14.53.

Conclusion

Efficient synthetic routes to antalarmin isotopomers **1** and **2** were successfully developed and carried out. These routes provided the desired compounds in reasonable chemical yields with high isotopic purity. Pharmacokinetic studies using these non-peptide stable isotope CRHR1 antagonists will be reported in due course.

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