NUCLEAR MAGNETIC RESONANCE STUDIES OF CANNABINOID RECEPTOR SITE PEPTIDES

Vicky L. H. Bevilacqua, John H. Brown, Jeffrey S. Rice
U. S. Army SBCCOM, Aberdeen Proving Ground, MD 21010-5424, USA

Anna M. Hutchings, Travis P. Albright, Dow P. Hurst and Patricia H. Reggio
Department of Chemistry & Biochemistry, Kennesaw State University, Kennesaw, GA, 30144, USA

ABSTRACT

Recent mutagenesis and chimera studies have implicated the second extracellular loops (E2) of CB1 and CB2 in ligand binding. As a contribution toward understanding the role of E2 in the interactions of CB1 and CB2 with cannabinoids, we have initiated structure studies on two E2 peptide analogues in the absence and presence of SDS using nuclear magnetic resonance (NMR) spectroscopy. Based on alpha proton chemical shifts, CB2-E2 and CB1-E2 have random coil conformations in an aqueous environment. The N-terminal half (residues E_1-S_8) of CB1-E2 in SDS micelles contains an alpha-helical stretch. CB2-E2 in the presence of SDS consists of at least two equally populated stable conformations, each having alpha proton chemical shifts consistent with random coil conformations. Preliminary simulated annealing calculations suggest that the E2 peptides in both the absence and presence of SDS contain a well-defined region that includes the CSXXXP sequence shared by the cannabinoid receptors and several orphan receptors. The precise conformation of the CSXXXP region depends on the peptide and the solvent system.

INTRODUCTION

The cannabinoid receptor subtypes CB1 and CB2 are part of the G-protein coupled receptor (GPCR) superfamily. GPCRs activate cellular signal transduction mechanisms and share a similar topology (Fig. 1), having an extracellular N-terminus, a 7 transmembrane (7-TM) spanning helix bundle, and an intracellular C-terminus. The ligand binding sites are thought to be within the 7-TM bundle, on the extracellular loops, or to include portions of both regions.

CB1 and CB2 share 44% sequence identity overall and 68% sequence identity within the 7-TM helices. The helix net of the human CB1 receptor is shown in Figure 2.

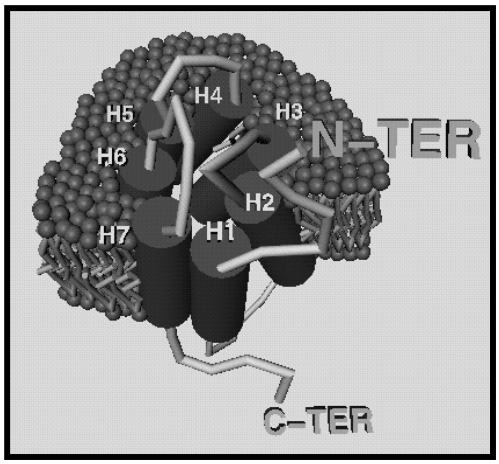


Figure 1. 7-TM helix bundle.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUL 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Nuclear Magnetic Resonance Studies Of Cannabinoid Receptor Site Peptides	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U. S. Army SBCCOM, Aberdeen Proving Ground, MD 21010-5424, USA**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001523., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

Recent binding studies have indicated some involvement of the CB1 and CB2 second extracellular loops (E2 loops) in ligand binding. Shire *et al*[1] prepared chimeras in which various regions were swapped between CB1 and CB2 and the binding was assayed. E2 was the only loop found to have an impact on binding. Table I shows a summary of their results for this loop. In the CB1/$E2_{CB2}$ and CB2/$E2_{CB1}$ chimeras, the binding of the agonist CP55,940 is affected, but not that of the CB1 antagonist SR141716A (Fig. 3 and Table 1).

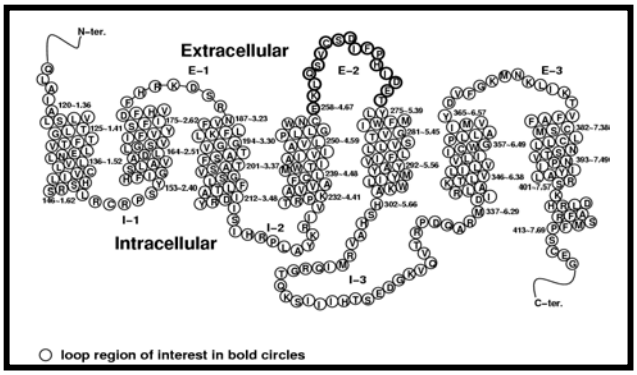


Figure 2. Helix net of the human CB1 receptor.

We are, thus, carrying out nuclear magnetic resonance (NMR) structure studies on analogue peptides of the E2 loops (Fig. 4) in the interest of correlating E2 structure with possible function in ligand binding. The analogue peptides have the conservative substitution of serine for cysteine to avoid inter-peptide disulfide bond formation. Each peptide is acetylated at the N-terminus and amidated at the C-terminus. The methods used here will be of importance for future structural work on peptide and protein toxins and their interaction with target receptors and, therefore, the E2 peptides serve as model systems for such research.

Table 1. Shire *et al*[1] E2 Chimera Binding

		CP 55,940 Kd (nM)	SR141716A Kd (nM)
CB1	wt	0.4	0.38
	$E2_{CB2}$	no binding	0.44
CB2	wt	0.2	no binding
	$E2_{CB1}$	no binding	no binding

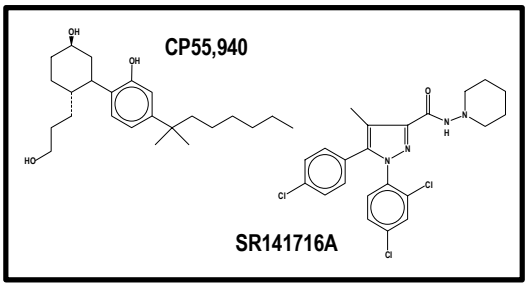


Figure 3. CP55,940 and SR141716A.

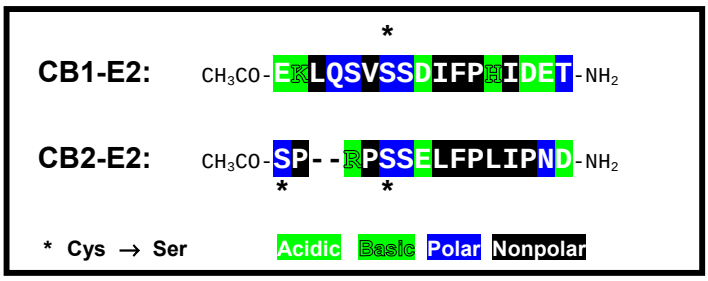
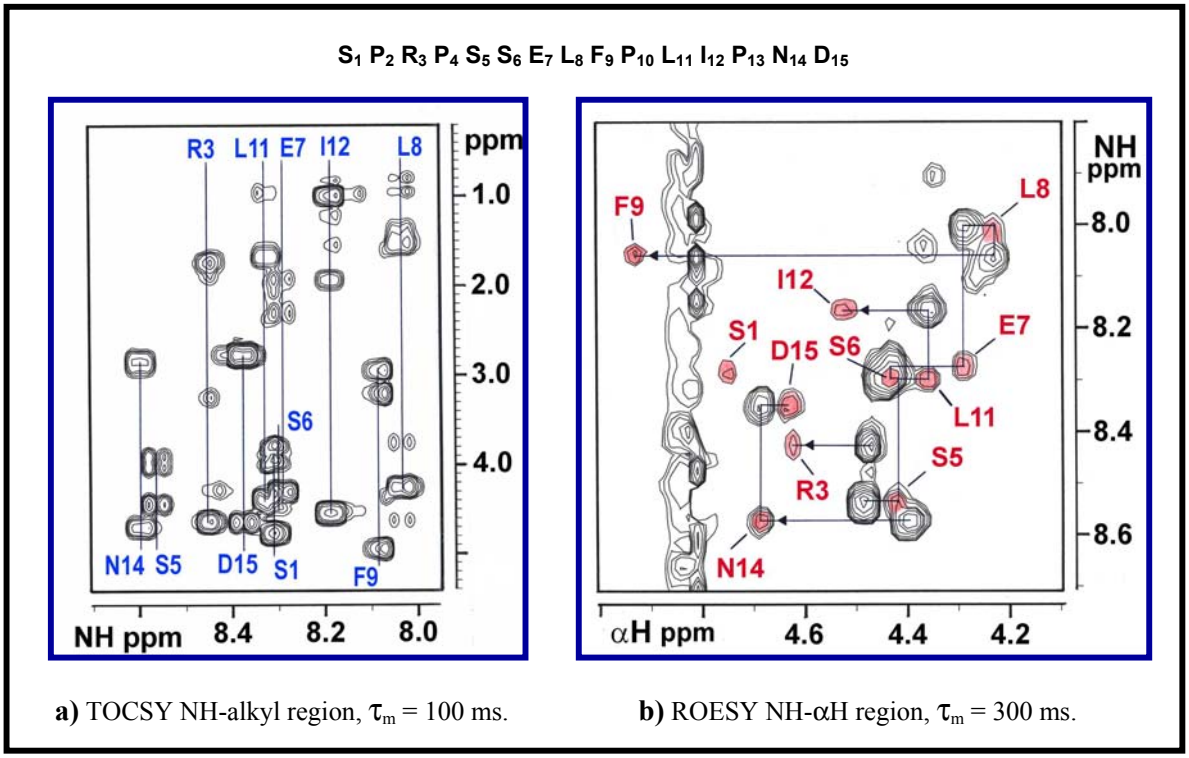


Figure 4. CB1- and CB2-E2 analogue peptides.

METHODS & RESULTS

2D NMR

Peptides were synthesized by the Emory University Microchemicals Facility. Total Correlation Spectroscopy[2] (TOCSY) and Nuclear Overhauser Effect Spectroscopy[2] (NOESY) or Rotating Frame Nuclear Overhauser Effect Spectroscopy[2] (ROESY) experiments were carried out for CB1-E2 in the presence of SDS and CB2-E2 in the presence and absence of deuterated sodium dodecyl sulfate (SDS) (pH range 4.8-5.69). Samples were buffered with deuterated sodium acetate. Experiments were carried out at 500 MHz on a Bruker DRX spectrometer or at 800 MHz on a Varian Inova spectrometer.



a) TOCSY NH-alkyl region, τ_m = 100 ms. **b)** ROESY NH-αH region, τ_m = 300 ms.

Figure 5. CB2-E2 spectra (no SDS), 10 mM, pH 4.8, H_2O:D_2O = 7:3, 300 K, 500 MHz.

Spin systems were identified using TOCSY spectra. Selected results for CB2-E2 in the absence of SDS (pH 5.69) are shown in Figure 5.a. Backbone assignments were determined by comparing ROESY (no SDS) or NOESY (with SDS) spectra with TOCSY data. The backbone assignment trace for CB2-E2 (no SDS) is shown in Figure 5.b. Stereospecific assignments were not carried out.

Selected results from assignments for CB2-E2 in SDS (pH 5.69) are shown in Figure 6. Two sets of equal-intensity peaks indicate at least two conformations exist for CB2-E2 in SDS. The two conformations are arbitrarily labeled Conf. 1 and Conf. 2.

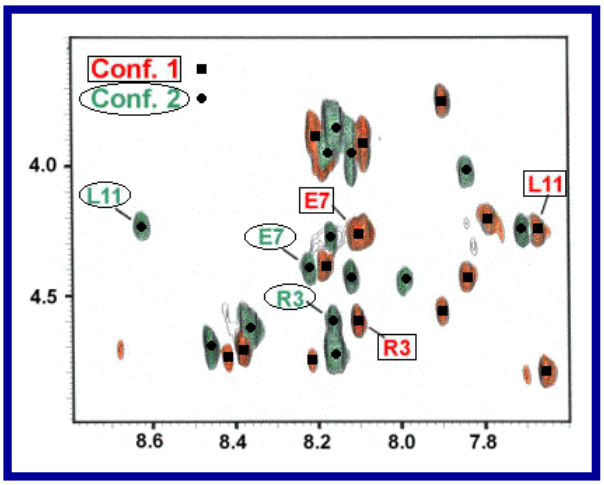


Figure 6. CB2-E2/SDS TOCSY NH-αH region, 2 mM, pH 5.7, SDS = 61 mM, H_2O:D_2O = 7:3, 298 K, 800 MHz, τ_m = 80ms.

STRUCTURE CALCULATIONS

Structure calculations were carried out with X-PLOR 3.1[3]. For each E2 calculation, fifty starting structures were minimized using a random seed followed by molecular dynamics at 300 K and re-optimization. Simulated annealing was carried out by heating to 2000 K (1000 steps) and cooling to 100 K (1000 steps) with a timestep of 0.0003 ps. The number of NOEs included in structure calculations are tabulated in Table 2. Structures having NOE violations greater than or equal to 0.5 Å were rejected.

Table 2. Summary of NOEs Included in Structure Calculations

		Overall	N- & C-Terminus	CSXXXP
CB1-E2/SDS	**Intra-residue**	62	45	17
	Inter-residue	28	3	25
CB2-E2/SDS Conf. 1	**Intra-residue**	50	31	19
	Inter-residue	34	6	28
CB2-E2/SDS Conf. 2	**Intra-residue**	53	32	21
	Inter-residue	43	12	31
CB2-E2/H_2O	**Intra-residue**	36	24	12
	Inter-residue	26	11	15

NOEs for significantly overlapped peaks were not included.

Accepted structures were overlaid according to backbone torsion angles within the CSXXXP region using XCluster[4] and viewed using Macromodel[5] as shown in Figure 7 for CB1-E2 in SDS, CB2-E2 in H_2O and CB2-E2 Conf. 1 in SDS. Calculations for CB2-E2 Conf. 2 are in progress. The observed NOEs and H_α chemical shifts for F_9 and L_{11} imply that P_{10} may be in the *cis*- conformation in Conf. 2 while it is in the *trans*- conformation in Conf. 1.

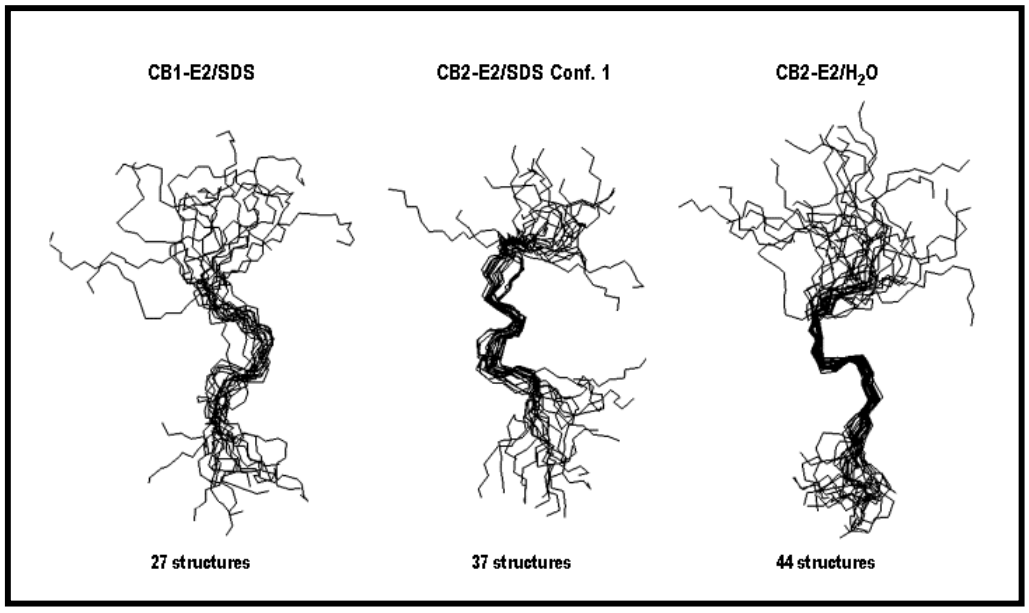


Figure 7. Overlaid structures from X-PLOR calculations on E2 analogue peptides.

LOOP STRUCTURE CALCULATIONS

The distance between the E2 N- and C-termini α protons is expected to be approximately 13 Å based on our current models for the CB1 and CB2 helix bundles. Studies suggest that a disulfide bridge links Cys 4.66 and the cysteine of the CSXXXP motif.

```
          4.66      CSXXXP
           ↓         ↓
CB1       CEKLQSVCSDIFPHIDET
CB2       CCP--RPCSELFPLIPND
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A second set of calculations was carried out on these sequences to determine the feasibility of formation of loops having the same structural constraints found for the analogue peptides in solution with the added requirements expected to be present in an intact receptor. The following structural requirements were, thus, included in the calculations:

1) a disulfide bridge linking the two Cys residues,
2) a 13 Å distance constraint between the N- and C-termini α protons,
3) the same NOE constraints as used for the analogue peptides in solution above.

As before, structures having NOE violations greater than or equal to 0.5 Å were rejected. Overlaid structures calculated using NOEs for CB1-E2 in SDS and for CB2-E2 in H_2O are shown in Figure 8.

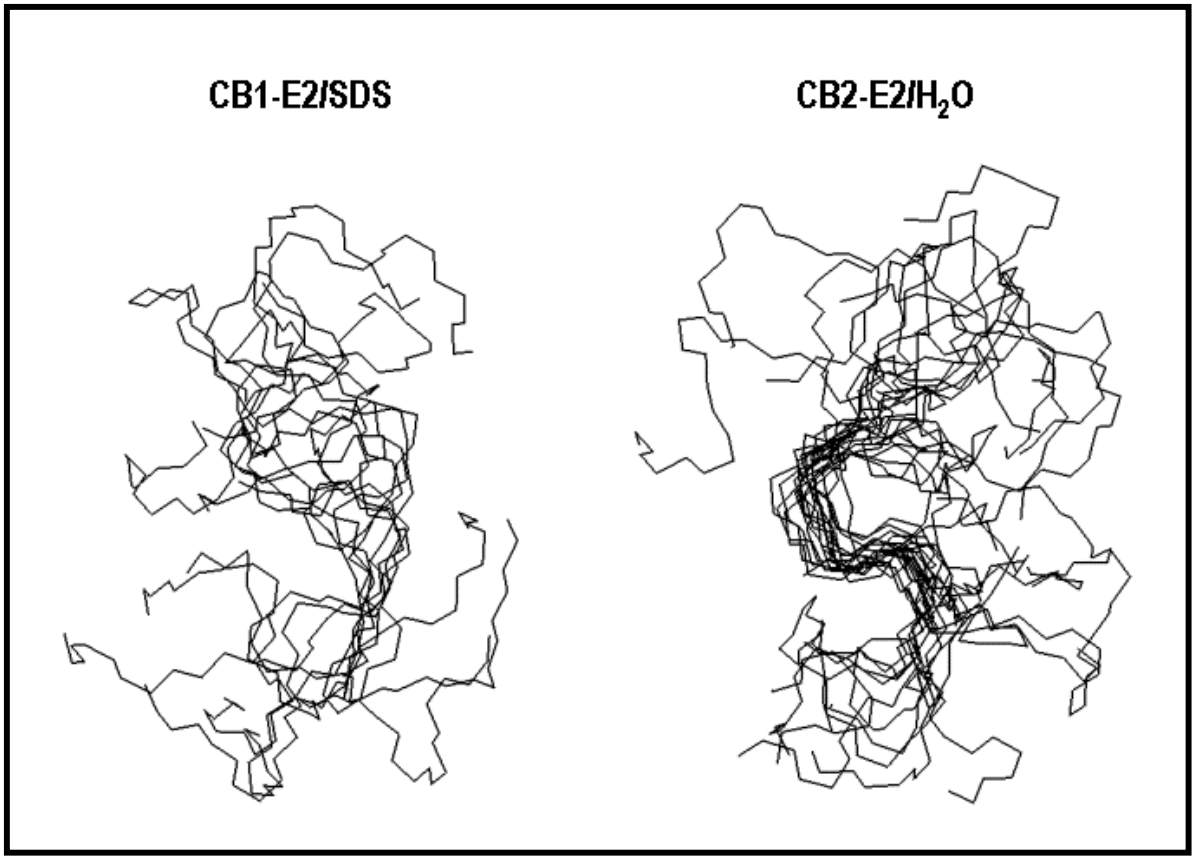

Figure 8. Overlaid structures from calculations having modeled disulfide bridges and N- to C terminus distance constraints.

CONCLUSIONS

STRUCTURE CALCULATIONS

The CSXXXP motif of each peptide has well-defined structure in the absence and presence of SDS micelles. The precise structure of the turn depends on the specific peptide sequence and the solvent system. Conf. 1 of CB2-E2 in the presence of SDS contains a loop, while CB2-E2 in H_2O and CB1-E2 in SDS have turns or bends. The fact that the CSXXXP motif is well-defined in the E2 analogue peptides apart from the receptors, and in more than one environment, indicates some importance for a CSXXXP tight turn in the function of receptors which contain this sequence.

LOOP STRUCTURE CALCULATIONS

Incorporating a disulfide bridge and an N- to C-terminus distance constraint along with NOE constraints observed for CB1-E2/SDS or CB2-E2/H_2O yielded structures without NOE violations. Although speculative, these structures represent conformations that could be reasonably adopted by loops as part of intact receptors. Similar calculations using NOE constraints observed for CB2-E2/SDS Conf. 1 gave no acceptable structures. Thus, the well-defined region of structure for this analogue peptide in solution does not represent a possible stable conformation for this E2 loop in the intact receptor. The fact that the CSXXXP motif structural features are readily preserved for two of the 3 cases when the disulfide bridge and N- to C-terminus constraint are included further supports the significance of this motif in the structure of the E2 loops.

IMPLICATIONS

Several orphan receptors share the CSXXXP motif[6]. A subset of these receptors has E2 loops of similar length to those of CB1 and CB2 and a cysteine at position 4.66. It is possible that the disulfide bridge stabilizes the structure in the N-terminal half while the CSXXXP motif provides the stabilizing feature for the C-terminal half of these short E2 loops. Furthermore, sphingosine 1-phosphate has recently been determined to be the endogenous ligand for EDG-1[7], the orphan receptor most homologous to CB1 and CB2. Thus, a tight turn within the CSXXXP motif may facilitate binding of lipid-like ligands to receptors with short E2 loops.

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CB1      CEKLQSVCSDIFPHIDET
CB2      CCP--RPCSELFPLIPND
GPR12    CLRDESTCSVVRPLTKNN
GPR6     CLAERAACSVVRPLARSH
GPR3     CLDGLTTCGVVYPLTKNN
rCNL3    CLADRASCSVVRPL-TRS
GPCR21   CRDGLTTCGVVYPL-SKN
6-7      CLRDESTCSVVRPL-TKN
edg-1    CISALSSCSTVLPLYHKH
ARG16    CLDHLEACSTVLPLYAKH
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ACKNOWLEDGEMENTS

We thank Dr. Leslie Gelbaum (Georgia Institute of Technology, Atlanta, GA) and Dr. John Glushka (Complex Carbohydrate Research Center, Athens, GA) for help with NMR experiments. Support was also contributed through KSU PIs: NSF DUE-9452027 (VLHB), NIDA DA-03934 (PHR), Kennesaw State University Mentor-Protégé Program (VLHB).

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