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Synthesis and biological evaluation of new salvinorin A analogues incorporating natural amino acids

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Abstract

The synthesis and in vitro evaluation of a new series of salvinorin A analogues substituted at the C(2) position with natural amino acids is reported. Compound 12, containing Val, displayed high affinity and full agonist activity at the kappa-opioid receptor. Analogues with bulky and/or aromatic residues were inactive, showing the importance of size and electronegativity of C(2)-substituents for binding affinity of salvinorin A derivatives.

Keywords

Salvia divinorum; salvinorin B; neoclerodane diterpenoid; natural amino acids; opioid receptor ligands

A neoclerodane diterpenoid salvinorin A (SA) (**1**), the active ingredient of the Mexican plant *Salvia divinorum*, is a selective kappa-opioid receptor (KOR) agonist with potent *in vivo* activity (Fig. 1).¹ Recent reports point out a potential role in the treatment of functional gastrointestinal (GI) disorders, which is in accord with the traditional use of *S. divinorum* extracts. SA potently inhibits smooth muscle contractility and motility *in vivo* in the upper and lower GI, as well as influences the epithelial ion transport in a KOR-dependent manner under both physiological and pathophysiological conditions.² Pharmacokinetic studies in animals and in humans indicate that SA is rapidly degraded and that its *in vivo* effects are short-lasting, regardless of route of administration.³ Therefore numerous attempts have been made to synthesize SA derivatives with potent affinity at KOR and prolonged *in vivo* actions.² The structure-activity relationship (SAR) studies of SA have demonstrated that the size and electronegativity of the substituent at the C(2) position is critical for activity at opioid receptors. It was shown that SA derivatives with spatially restrained (3-4 carbon atoms) lipophilic groups at C(2) display highest binding affinity and potency at KOR.⁴⁻⁹ However, incorporation of non-lipophilic substituents, such as amines and amino acids also

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resulted in potent and selective KOR ligands.^{6,8} Interestingly, bulkier substituents tend to reduce KOR affinity and in some cases increase mu-opioid receptor (MOR) affinity.²

In an attempt to further investigate the role of C(2) in SA binding at KOR we synthesized a series of derivatives substituted in this position with natural non-aromatic and aromatic amino acids and evaluated their *in vitro* affinities and potencies. Previous studies showed that the introduction of an aromatic moiety in position 2 increased affinity at MOR.¹⁰ Therefore, N-(9-fluorenylmethoxycarbonyl) (Fmoc)-protected derivatives were also synthesized and their affinities compared with those of respective non-protected analogues.

SA (**1**) was isolated from the leaves of *S. divinorum* and purified as previously described.¹¹ Compound **1** was converted to salvinorin B (**2**), which served as the starting material for the preparation of the C(2)-modified analogs **3–14** (Table 1). Compounds **3–8** were synthesized from N-Fmoc-protected amino acids using N-methylmorpholine as a catalyst and TBTU as a coupling reagent (Scheme 1).¹² Analogs **9–14** were obtained from respective N-Fmoc-protected derivatives by deprotection with piperidine.^{13;14}

Spectral data (¹H NMR, ¹³C NMR, high-resolution mass) consistent with the proposed structures were obtained for all the compounds prepared in this study.

The KOR radioligand binding assays were conducted according to previously described procedures and the results are presented in Tables 2 and 3.¹⁵

[³⁵S]GTPγS binding assays at MOR, DOR and KOR were performed for analogue **12** and SA, which was used as a reference compound. The *in vitro* pharmacological data for SA and **12** are listed in Table 4.

Preliminary *in vitro* binding data in opioid receptor-expressing cells showed that the introduction of N-Fmoc-protected amino acids at the C(2) position of SA resulted in loss of affinity at KOR, depending on the amino acid incorporated and the radiolabelled ligand used (Table 2). Only in the case of the Val-substituted analogue (**12**) did the removal of the Fmoc protecting group significantly increase its ability to displace selective ligands at KOR binding sites, making it almost equipotent with the parent compound, SA. Interestingly, the Ala-derivative (**10**) displayed good affinity at KOR against [³H]U69,593, but did not displace KOR antagonist binding, as assessed with [³H]diprenorphine. Concentration-response curves showed that analogue **12** had high affinity and selectivity at KOR (K_i values of 42.0 ± 2.05 and 0.75 ± 0.62 nM for **12** and SA, respectively; Table 3), while the affinity of **10** was in a μM range (Table 3). To determine the agonist activity of **12** at KOR, [³⁵S]GTPγS binding assays were performed. The potency of Val-substituted derivative (**12**) was lower than that of the parent compound (see Table 4); however, the novel analogue was a full agonist at KOR (Table 4).

Amino acid analogues of SA substituted at the C(2) position were reported earlier, but only N-Boc-Gly-derivative displayed good affinity at KOR (90 ± 10 nM).⁸ The introduction of N-Ac-Gly, N,N-diMeGly and N,N-diMeGly gave inactive compounds, while the Gly and Sar analogues were unstable.⁶ In our study the modification with Val resulted in a potent and selective KOR agonist, with affinity in the nM range. These data suggest that the C(2) position in the structure of SA is important for its interaction with the opioid receptors and that only small, non-aromatic amino acids fit into the binding pocket of KOR.

This is in good agreement with a recently published paper by McGovern et al. on molecular modelling of C(2)-analogues of SA.¹⁶ A region of bulk tolerance, falling within a receptor hydrophobic pocket, was observed around the C(2) position, extending approximately three carbon atoms in length from the carbonyl carbon. It has also been shown that the methyl and

carbonyl groups, as well as a hydrophobic moiety at a terminal position of the C(2) substituent are important for optimal ligand affinity. Interestingly, the structure of Val-derivative, which seems to be well-tolerated by the putative binding pocket, accommodates all above mentioned criteria.

Unexpectedly, in the study of Beguin et al.⁶ and in our assays, the Ala-derivative did not displace [³H]diprenorphine from its binding at KOR. However, here we observed that the Ala-substituted analogue displayed μ M affinity against [³H]U69,593, clearly demonstrating that the choice of the radiolabeled compound can influence the reported binding affinity data.

In conclusion, we were able to obtain a series of novel amino acid analogues of SA, of which the Val-derivative displayed highest affinity at KOR, comparable to that of the parent compound. Due to relative ease of our approach and the availability of reagents the synthetic strategy used in this study may result in a vast library of analogues and could become the method of choice in preparation of novel derivatives based on SA structure.

Acknowledgments

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12. General procedure for synthesis of N-Fmoc-amino acid analogues of salvinorin A (**3-8**): To a stirred solution of **2** (20 mg, 0.05 mmol, 1 equiv) in CH₂Cl₂ (2 ml), the N-Fmoc-amino acid (3 equiv), and N-methylmorpholine (3 equiv) was added TBTU (3 equiv). The solution was stirred at 50°C (24 h), concentrated under reduced pressure and purified by column chromatography (SiO₂; 2:1, hexane:EtOAc) to obtain the desired product.
13. General procedure for deprotection of N-Fmoc-amino acid analogues of salvinorin A (**9-14**): respective N-Fmoc-amino acid derivative of salvinorin A (1 equiv) was dissolved in CH₂Cl₂ and piperidine (3 eq) was added. The solution was stirred at RT (1.5 h), concentrated under reduced

pressure and purified by column chromatography (SiO₂; 2:1, hexane:EtOAc) to obtain the desired product.

14. Synthesis of 2-(2'-(*S*)-amino-3-methylbutanoate) salvinorin B (**12**): yield 76.9% starting from **2**, a white solid, δ 0.62 (1:1, hexane:EtOAc); ¹H NMR (400 MHz, CDCl₃) δ 1.02-1.04 (d, *J* = 6.8Hz, 3H), 1.07-1.10 (d, *J* = 6.8Hz, 3H), 1.14 (s, 3H), 1.47 (s, 3H), 1.57-1.75 (m, 4H), 1.77-1.84 (m, 1H), 2.07-2.11 (dd, *J* = 10.8, 1.6Hz, 1H), 2.16-2.21 (m, 2H), 2.30-2.36 (m, 2H), 2.48-2.55 (dd, *J* = 13.4, 5.2Hz, 1H), 2.75-2.80 (dd, *J* = 13.0, 3.5Hz, 1H), 3.44-3.46 (d, *J* = 4.4Hz 1H), 3.75 (s, 3H), 5.17-5.22 (dd, *J* = 12.6, 7.5Hz, 1H), 5.53-5.57 (dd, *J* = 11.6, 5.1 Hz, 1H), 6.41 (s, 1H), 7.41 (t, *J* = 2.7Hz, 1H), 7.45 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) 15.2; 16.4; 16.9; 17.0; 18.1; 19.4; 30.9; 34.4; 35.5; 38.2; 42.1; 43.4; 51.4; 52.0; 53.6; 64.0; 71.2; 75.2; 108.5; 125.5; 139.5; 143.7; 171.1; 171.6; 172.1; 202.0 ppm; HRESIMS *m/z* [M+H]⁺ 490.08 (calcd for C₂₆H₃₅NO₈ 489.56).
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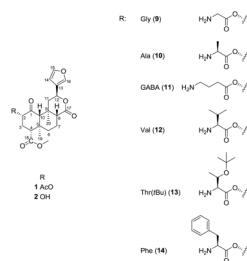
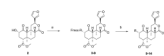


Figure 1.
Structure of salvinorin A (1), salvinorin B (2) and new amino acid analogues.

**Scheme 1.**

Synthesis of amino acid analogues of salvinorin A. Reagents and conditions: a) salvinorin B (**2**), appropriate N-Fmoc-amino acids, TBTU, N-methylmorpholine, CH₂Cl₂, b) deprotection with piperidine in CH₂Cl₂.

Table 1

Physicochemical data for amino acid analogues of salvinorin A

Analogue No.	Amino acid residue	TLC	HPLC t_r^c [min]	FAB-MS	
				Formula	MW [M+H] ⁺
3	Fmoc-Gly	0.54 ^a	17.6	C ₃₈ H ₃₉ NO ₁₀	669.72
4	Fmoc-Ala	0.75 ^a	19.0	C ₃₉ H ₄₁ NO ₁₀	684.37
5	Fmoc-GABA	0.39 ^a	17.7	C ₄₀ H ₄₃ NO ₁₀	698.30
6	Fmoc-Val	0.84 ^a	17.8	C ₄₁ H ₄₅ NO ₁₀	712.31
7	Fmoc-Thr(tBu)	0.62 ^a	19.2	C ₄₄ H ₅₁ NO ₁₁	769.87
8	Fmoc-Phe	0.56 ^a	19.3	C ₄₅ H ₄₅ NO ₁₀	759.83
9	Gly	0.56 ^b	12.6	C ₂₃ H ₂₉ NO ₈	447.48
10	Ala	0.65 ^b	12.8	C ₂₄ H ₃₁ NO ₈	461.50
11	GABA	0.77 ^b	13.1	C ₂₅ H ₃₃ NO ₈	475.53
12	Val	0.62 ^b	14.1	C ₂₆ H ₃₅ NO ₈	489.56
13	Thr(tBu)	0.67 ^b	18.7	C ₂₉ H ₄₁ NO ₉	547.64
14	Phe	0.61 ^b	17.6	C ₃₀ H ₃₅ NO ₈	537.60

^a R_f values for TLC in hexane:EtOAc (1:1).^b R_f values for TLC in CH₂Cl₂:MeOH (49:1).^c HPLC elution on a Waters RP C₁₈ column (7.8 × 100 mm, 5 μm) using the solvent system of water (A)/ acetonitrile (B) and a linear gradient of 30-100% solvent B over 25 min at flow rate of 1 mL/min.

Table 2

Opioid receptor binding data for amino acid analogues of salvinorin A

Analogue No.	Amino acid residue	MOR [%] ^a	DOR [%] ^b	KOR [%] ^c	KOR [%] ^d
3	Fmoc-Gly	20	16	4	10
4	Fmoc-Ala	24	15	21	0
5	Fmoc-GABA	27	24	10	0
6	Fmoc-Val	15	22	39	0
7	Fmoc-Thr(<i>t</i> Bu)	18	8	28	12
8	Fmoc-Phe	0.5	3	0	34
9	Gly	-5	17	41	0
10	Ala	-1.6	18	82	0
11	GABA	1	28	48	0
12	Val	5	48	103	91
13	Thr(<i>t</i> Bu)	2	11	6	0
14	Phe	-6	13	19	0
DAMGO		100	-	-	-
Natrinole		-	100	-	-
SA		-	-	100	100

Data are mean of three experiments performed in duplicate. All compounds were tested at a concentration of 10 μ M.

^aDisplacement of [³H]DAMGO in MOR-expressing cells, presented as % effect of respective control (DAMGO, 10 μ M).

^bDisplacement of [³H]DADLE in DOR-expressing cells, presented as % effect of respective control (DADLE, 10 μ M).

^cDisplacement of [³H]U69,593 in KOR-expressing cells, presented as % effect of respective control (SA, 10 μ M).

^dDisplacement of [³H]diprenorphine in KOR-expressing cells, presented as % effect of respective control (SA, 10 μ M).

Table 3

Opioid receptor binding affinity and selectivity for selected amino acid analogues of salvinorin A

Analyte No.	Amino acid residue	K_i^a [MOR, nM]	K_i^b [DOR, nM]	K_i^c [KOR, nM]	MOR/KOR	DOR/KOR
10	Ala	> 10,000	> 10,000	3784 ± 1284	>2.64	>2.64
12	Val	> 10,000	> 10,000	42.0 ± 2.05	>238	>238
SA	-	> 10,000	> 10,000	0.75 ± 0.62^d	> 10,000	> 10,000

Data are mean of three experiments performed in duplicate.

^aDetermined against [³H]DAMGO

^b[³H]DADLE

^c[³H]U69,593.

^dData from ref.¹⁵

Table 4Agonist activity of analogue **12** in [³⁵S]GTP γ S binding assay

Analogue No.	Amino acid residue	EC ₅₀ [nM]	E _{max} [%]	Relative E _{max}
12	Val	213 ± 15.0	828 ± 275	117.74
SA	-	0.2 ± 0.1	703 ± 179	100.00

Data are mean ± SEM of four experiments.