Isolation and Chemical Modification of Clerodane Diterpenoids from *Salvia* Species as Potential Agonists at the κ-Opioid Receptor

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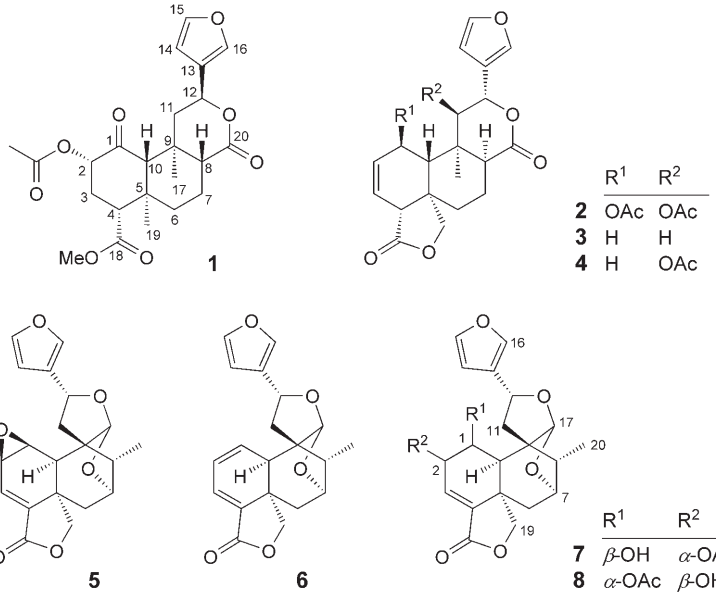
The clerodane diterpenoid salvinorin A (**1**), the main active component of the psychotropic herb *Salvia divinorum*, has been reported to be a potent agonist at the κ-opioid receptor. Computer modeling suggested that splendidin (**2**) from *S. splendens*, as well as related compounds, might possess similar activities. In the present study, this hypothesis was tested by determination of the binding properties of a series of structural congeners, compounds **2**–**8**, at the μ-, δ-, and κ-opioid receptors. However, none of these compounds showed significant binding to any of the opioid-receptor subtypes, thus disproving the above hypothesis.

The novel compounds **7** and **8** were obtained semi-synthetically by selective modification of salvifarin (**5**), isolated from *Salvia farinacea*, upon epoxide-ring opening with AcOH in the presence of indium(III) triflate. Also, the X-ray crystal structure of salvifaricin (**6**; *Fig.*), obtained from *S. farinacea*, was determined for the first time and used, in combination with in-depth NMR experiments, to elucidate the absolute configurations of the new products. Our experiments demonstrate that the relatively well-accessible diterpenoid **6** could be used as starting material for future studies into the structure–activity relationship at the κ-opioid receptor.

Introduction. – *Salvia divinorum*, also referred to as 'magic mint' or 'holy mint', is a psychotropic plant first described in the 1960s [1]. A number of clerodane diterpenoids have been isolated from this species [2], of which salvinorin A (**1**) was identified as the major active constituent [3][4]. This compound was subsequently shown to act as an agonist at the κ-opioid receptor, the first non-nitrogenous compound to do so [5][6]. Modeling studies with **1** led to the hypothesis that splendidin (**2**), another clerodane diterpenoid from *Salvia splendens*, might also possess κ-opioid activity [5]. However, the study of structure–activity relationships (SAR) by a number of groups using modified derivatives of **1** have shown particular structural requirements lacking in **2**, including an acetyl (Ac) group or another small substituent at the C(2) atom [7], as well as *trans*-fused *B*/*C* rings, with a β-orientated H-atom at C(8) [8].

The purpose of the present study was to test whether or not splendidin (**2**) is, indeed, a κ-opioid agonist, and to compare its biological properties with those of other diterpenoids from *S. splendens*, including salviarin (**3**) and splenolide B (**4**). We also isolated the clerodanes salvifarin (**5**) and salvifaricin (**6**) from *S. farinacea*, and tested them together with the semi-synthetic derivatives **7** and **8**.



Results and Discussion. – 1. *X-Ray Crystal Structure of* **6**. The known compounds **2**–**6** were isolated from the corresponding natural sources, and identified by comparison of their spectroscopic data with those published previously. The X-ray crystal structures of **1** [3], **3** [9], and **5** [10] have been published before, and a single-crystal X-ray diffraction analysis of salvifaricin (**6**) was carried out in this study (*Figure*), which confirmed that the configuration of **6** is the same as in salvifarin (**5**).

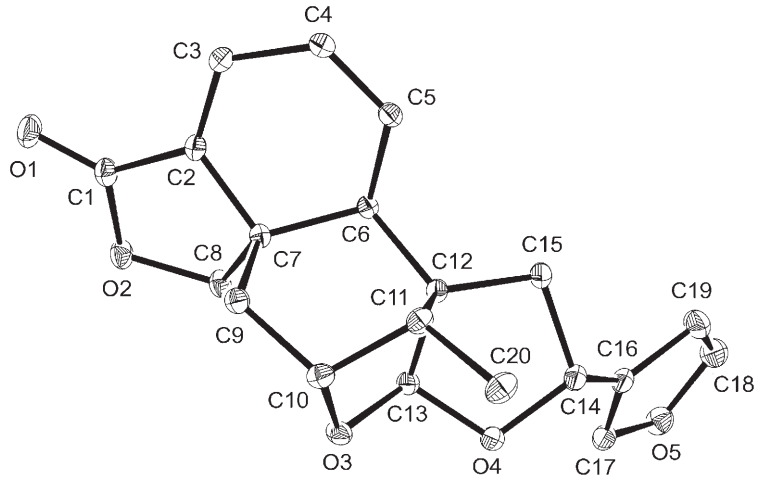


Figure. *X-Ray crystal structure of salvifaricin* (**6**). Arbitrary atom numbering (different from diterpenoid numbering). Ellipsoids are represented at the 30% probability level, and H-Atoms are omitted for clarity.

2. *Epoxide-Ring Opening of* **5** *and Structural Analysis of the Resulting Congeners.* Starting from salvifarin (**5**), we synthesized the two new compounds **7** and **8** by *Lewis* acid-catalyzed epoxide-ring opening with AcOH. As *Lewis* acid, indium trifluoromethanesulfonate (indium triflate) was chosen because of the previously reported effectiveness of In^{III} in promoting the selective ring opening of epoxides and its wide tolerance to other functional groups [11]. The resulting two geometric isomers, **7** and **8**, were characterized by MS and NMR, and their structures were confirmed by comparison of their ^{13}C- and ^{1}H-NMR data with those of **5** (*Tables 1* and *2*, resp.).

Table 1. *^{13}C-NMR Data of* **5**–**8**. Recorded at 100 MHz in $CDCl_3$; δ in ppm.

Position	**5**	**7**	**8**	Position	**5**	**7**	**8**
1	58.7	70.6	73.4	12	75.7	76.0	75.9
2	46.2	71.4	66.3	13	128.6	128.9	128.3
3	129.9	129.3	131.9	14	108.2	108.2	108.2
4	137.6	138.0	138.5	15	143.8	143.7	143.9
5	58.5	58.5	58.2	16	138.8	138.3	136.6
6	40.6	38.9	37.9	17	110.1	110.7	110.7
7	87.2	87.2	86.9	18	169.3	168.7	169.2
8	39.1	40.0	39.8	19	79.8	80.7	80.4
9	44.0	43.4	43.4	20	14.9	14.9	14.6
10	45.7	46.4	43.8	*Me*CO	–	21.0	21.5
11	38.8	39.4	38.2	Me*C*O	–	170.2	170.1

A critical element in the discrimination of the two regioisomers **7** and **8** was the accurate ^{1}H- and ^{13}C-NMR assignment of H–C(1) and H–C(2). In the parent compound **5**, the two α-H-atoms at these positions resonate as an unresolved *multiplet* at δ(H) 3.54, showing HMQC correlations with the respective C-atom signals at δ(C) 58.7 and 46.2. HMBC Experiments showed a correlation between the signal at δ(C) 46.2 and δ(H) 7.01 (H–C(3)). Thus, the signal at δ(C) 46.2 was assigned to C(2). HMBC also showed a correlation between δ(C) 58.7 and δ(H) 2.07 (H–C(10)); thus, this signal was assigned to C(1).

High-resolution CI-MS analysis of the quasi-molecular $[M+NH_4]^+$ ion of **7** indicated the molecular formula $C_{22}H_{24}O_8$, which confirmed the addition of AcOH to **5**. The ^{13}C-NMR spectrum of **7** (*Table 1*) was very similar to that of **5**, except for additional peaks for an AcO moiety at δ(H) 21.0 (Me) and 170.2 (C=O). At the same time, the signals for C(1) and C(2) were shifted to δ(C) 70.6 and 71.4, respectively. HMQC Analysis showed that these C-atoms were attached to H-atoms resonating at δ(H) 4.15 and 5.24, respectively. These could readily be located by COSY coupling of δ(H) 4.15 to H–C(10) at δ(H) 2.06, and of δ(H) 5.24 to H–C(3) at δ(H) 6.60, supported by an HMBC interaction of δ(C) 70.6 with H–C(10). Since the strongly deshielded methine at δ(H) 5.24 was likely to correspond to the acetylated position, we then placed the AcO group at C(2) and the OH function at C(1).

The configurations at C(1) and C(2) of **7** were determined by NOESY analysis, which showed interactions between δ(H) 4.15 (H–C(1)) and 2.06 (H_α–C(10)), as well as between δ(H) 5.24 (H–C(2)) and 2.94 (H_β–C(8)), in accord with the crystal structures of **5** and **6** (*Fig.*), in which H–C(8) is above the β-face of the *A*-ring.

Table 2. *^{1}H-NMR Data of* **5**–**8**. Recorded at 400 MHz in $CDCl_3$; δ in ppm, J in Hz.

Position	**5**	**7**	**8**
1	3.54 (*m*)	4.15 (*dd*, *J*=5.4, 3.9)	5.25 (*m*)
2	3.54 (*m*)	5.24 (*dd*, *J*=3.9)	4.25 (*m*)
3	7.01 (*t*, *J*=2.0)	6.60 (*d*, *J*=3.9)	6.68 (*d*, *J*=3.8)
6	2.16 (*dd*, *J*=14.1), 1.42 (br. *d*, *J*=14.1)	2.26 (*dd*, *J*=14.2, 3.9), 1.51 (*d*, *J*=14.2)	2.29 (*d*, *J*=13.4), 1.48 (br. *d*, *J*=13.4)
7	4.48 (br. *d*, *J*≈3.7)	4.51 (*d*, *J*=3.9)	4.54 (*m*)
8	2.65 (*q*, *J*=7.0)	2.94 (*q*, *J*=7.0)	2.42 (*q*, *J*=7.2)
10	2.07 (br. *s*)	2.06 (*d*, *J*=5.4)	2.27 (*d*, *J*=4.8)
11	2.88 (*dd*, *J*=7.8, 5.3), 1.97 (*dd*, *J*=7.8, 5.3)	2.88 (*dd*, *J*=7.9, 5.9), 1.80 (*dd*, *J*=7.9, 5.9)	2.67 (*dd*, *J*=7.7, 5.3), 1.84 (*dd*, *J*=7.7, 5.3)
12	5.35 (*t*, *J*=7.8)	5.28 (*t*, *J*=7.9)	5.28 (*t*, *J*=7.7)
14	6.30 (*m*)	6.30 (*m*)	6.28 (*m*)
15	7.40 (*m*)	7.39 (*m*)	7.38 (*m*)
16	7.37 (*m*)	7.35 (*m*)	7.35 (*m*)
17	5.26 (*s*)	5.26 (*s*)	5.18 (*s*)
19	4.90 (br. *d*, *J*=8.2), 3.90 (*dd*, *J*=8.2)	4.99 (*d*, *J*=7.8), 3.93 (*dd*, *J*=7.8)	4.99 (*d*, *J*=7.2), 3.91 (*d*, *J*=7.2)
20	1.36 (*d*, *J*=7.0)	1.36 (*d*, *J*=7.0)	1.30 (*d*, *J*=7.2)
Ac	–	2.10 (*s*)	2.12 (*s*)

Therefore, the configurations in **7** was deduced as 1β-OH and 2α-OAc, as predicted from the reaction mechanism.

The high-resolution ESI mass spectrum of **8** showed the $[M+Na]^+$ ion at m/z 439.1372, consistent with the molecular formula $C_{22}H_{24}O_8$. The ^{13}C-NMR spectrum (*Table 1*) was almost identical to that of **7**, but with slightly varied chemical shifts for C(1) and C(2) at δ(C) 73.4 and 66.3, respectively. HMQC Analysis showed that these signals correlated with those at δ(H) 5.25 and 4.25, respectively. The former showed a COSY correlation to δ(H) 2.27 (H−C(10)), and the latter correlated with δ(H) 6.68 (H−C(3)). This established that **8** had a 1-AcO and a 2-OH group. NOESY Data further showed a correlation between δ(H) 5.25 and 2.42 (H−C(8)), establishing that H−C(1) was on the β-face, the substituent, thus, being in α-position. The H-atom at C(2) showed no NOESY interactions, and, thus, the OH substituent was assigned to be β-orientated, in agreement with the expected reaction mechanism.

For both reaction products **7** and **8**, the rest of their ^{1}H- and ^{13}C-NMR signals were virtually unchanged compared to those of **5**, which confirmed that no other potential reactions, such as opening of the bicyclic acetal group or *Michael* addition to the olefin at C(3), had occurred under the conditions employed.

3. *Biological Studies.* Compounds **2**–**8** were tested for their binding affinity to the δ-, μ-, and κ-opioid receptors, using a competitive binding assay in *C6*μ, *C6*δ, and *CHO*κ cells, respectively, against the non-selective antagonist [^{3}H]diprenorphine [12]. None of these compounds showed greater than 50% inhibition of binding at 10 μM concentration, and, thus, no significant binding to any of these receptors.

Conclusions. – The previous hypothesis [5] that splendidin may be a good κ-opioid ligand is not supported by our studies. The model originally proposed [5] assumed H-

bonding interactions between a tyrosine OH group and the acceptor C=O groups at positions 1 and 18 of salvinorin (**1**), and between a glutamine residue and the furan O-atom of **1**. The same group later revised their model in the light of point-mutation studies to include hydrophobic interactions with the Me groups of the AcO and methyl ester functions of **1** [13]. A recent independent modeling study [14] confirms the importance of hydrophobic interactions in receptor binding by salvinorin A. Thus, although the H-bond acceptor sites of splendidin (**2**) can be mapped onto those of salvinorin A (**1**), the former lacks hydrophobic groups at the important positions 2 and 18.

Although salvifarin (**5**) and its semi-synthetic derivatives **7** and **8** were inactive as well, this work demonstrates the usefulness of this multifunctional diterpenoidal system in allowing selective chemical modification of the clerodane scaffold. As modeling studies become more sophisticated, there will be a continuing need for natural and semi-synthetic diterpenoids to explore the highly potent and selective κ-opioid agonist salvinorin (**1**) and the nature of its interactions with the receptor.

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Experimental Part

General. Hexane (reagent grade), AcOEt, CH_2Cl_2 (anal. grade), MeOH, MeCN (HPLC grade), H_2SO_4, and $NaHCO_3$ were supplied by *Fisher*. Vanillin and indium trifluoromethanesulfonate (triflate) were obtained from *Aldrich*. Silica-gel *60* for column chromatography (CC) and precoated silica-gel GF_{254} plates on Al backing for TLC were obtained from *Merck*. Semi-prep. HPLC was carried out with a *Constametric-4100* constant-volume pump, a *Jasco UV-1575* variable-wavelength UV detector (218 nm), and a *Phenomenex Synergi 4μ MAX-RP 8A* column (250 × 10.0 mm) maintained at 40° in a *SSI-505* LC column oven, eluting at a flow rate of 5 ml/min. Fractions were collected manually. IR Spectra were recorded on a *Perkin-Elmer RX1* FT-IR instrument; in cm^{-1}. NMR Spectra were obtained on *Jeol GX-270* or *Varian Mercury 400* spectrometers. Samples were dissolved in $CDCl_3$, and chemical shifts δ are reported in ppm rel. to internal Me_4Si. 2D-NMR (COSY, DEPT, HMQC, HMBC, NOESY) experiments were performed using normal pulse sequences. Low-resolution EI-MS (70 eV) and pos.-ion CI-MS (reagent ammonia) was carried out on a *Micromass Quattro-II* apparatus, and high-resolution (HR) MS was carried out on a *Finnigan MAT-900-XLT* mass spectrometer, both at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea. HR-ESI-MS was carried out on a *Bruker Daltonics MicroTOF* instrument (pos. mode); all values in *m/z*.

X-Ray Crystal Structure[1]). A single crystal of salvifaricin (**6**) was analyzed at 150(2) K using graphite-monochromated MoK_α radiation on a *Nonius Kappa-CCD* diffractometer. Details of the data collection and refinement are given in *Table 3*. The structure was solved with SHELXS-97, and refined by means of full-matrix least squares in SHELXL-97 [15].

Plant Material. Salvia splendens cv. Blaze of Fire, and *Salvia farinacea* cv. Victoria were grown in Bath, UK, from commercially available seeds. The plants were collected during the late flowering period, and air-dried.

Extraction and Isolation of Diterpenoids from S. splendens. Compounds **2**–**4** were isolated from the aerial parts of *S. splendens* by adaptation of reported methods [16]. Briefly, the powdered plant material (450 g dry weight) was macerated at r.t. with acetone (3 ×). The combined acetone extracts were

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[1]) The crystallographic data of **6** have been deposited with the *Cambridge Crystallographic Data Centre* as supplementary publication number CCDC-616424. Copies of the data can be obtained, free of charge, at http://www.ccdc.cam.ac.uk/data_request/cif.

Table 3. *Crystal Data and Structure Refinement for* **6**[1])

Formula	$C_{20}H_{20}O_5$
M_r [g/mol]	340.36
T [K]	150(2)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
a, b, c [Å]	11.1180(1), 11.3580(1), 12.6570(1)
$\alpha=\beta=\gamma$ [°]	90
V [Å^3]	1,598.30(2)
Z	4
D_c [g/cm^3]	1.414
μ [mm^{-1}]	0.101
$F(000)$	720
Crystal size [mm]	$0.50\times0.50\times0.50$
θ Range [°]	3.67–30.05
Miller indices	$-15\le h\le15;\ -15\le k\le15;\ -17\le l\le17$
Reflections collected	30,819
Independent reflections; R(int)	4,656; 0.0414
Reflections observed ($>2\sigma$)	4,482
Data/restraints/parameters	4656/0/228
Goodness-of-fit on F^2	1.045
Final $R1$, $wR2$ [$I>2\sigma(I)$]	0.0306, 0.0804
Final $R1$, $wR2$ (all data)	0.0321, 0.0818
Absolute structure parameter	0.0(5)
Max. and min. residual density [e/Å^3]	0.253, −0.188

evaporated to dryness at reduced pressure, and the resulting residue was re-extracted with AcOEt (3 ×). Evaporation of the solvent yielded a crude extract (5.6 g). A portion of this extract (2.5 g) was fractionated by CC (50 g SiO_2), eluting with a step gradient, starting with 5% AcOEt in hexane (1500 ml), followed by 10% AcOEt in hexane (1000 ml), and then in 5% steps up to 40% AcOEt in hexane (500 ml each). Fractions (*ca.* 45 ml) were collected and monitored by TLC and NMR spectroscopy. The fractions obtained with 40% AcOEt in hexane were combined and further purified by semi-prep. HPLC, eluting with 27% MeCN in H_2O. The HPLC retention times (t_R) were 70, 77, and 82 min for splendidin (**2**; 43 mg), salviarin (**3**; 33 mg), and splenolide B (**4**; 21 mg), resp.

Extraction and Isolation of Diterpenoids from S. farinacea. The powdered plant material (1500 g dry weight) was extracted as described above to yield a crude AcOEt-soluble extract (28 g). A portion of this extract (20 g) was separated by CC (100 g SiO_2), eluting with 5% AcOEt in hexane (1500 ml), followed by steps of 15, 30, 40, 45, 50, 70, 80, and 90% AcOEt in hexane (1000 ml each). Fractions (*ca.* 45 ml) were collected and monitored by TLC and NMR spectroscopy. On solvent evaporation, crystals separated from the fractions containing 45 and 50% AcOEt. These were collected and recrystallized from CH_2Cl_2/MeCN to yield salvifarin (**5**) (0.17 g) in the form of needle-shaped crystals. The mother liquors and the more-polar fractions were combined and concentrated, and the residue (3.4 g) was fractionated further by CC (100 g SiO_2), eluting with 1% MeOH in CH_2Cl_2 (9000 ml), followed by 2 and 3% MeOH in CH_2Cl_2 (1000 ml each). Fractions (*ca.* 60 ml) were collected and monitored by TLC and NMR spectroscopy. The fractions eluting between 3000 and 6000 ml of 1% MeOH in CH_2Cl_2 yielded a further crop of crystalline **5** (0.13 g after recrystallization). The fractions eluting between 6000 ml of 1% MeOH and of 400 ml of 3% MeOH in CH_2Cl_2 yielded crystalline salvifaricin (**6**; 12 mg after recrystallization from CH_2Cl_2/MeCN). Additional **6** was obtained by semiprep. HPLC of the mother liquors, eluting with 33% MeCN in H_2O (t_R 33 min).

Derivatization of Salvifarin (**5**). AcOH (50 mg, 0.8 mmol) and indium triflate (120 mg, 0.2 mmol) were added to a soln. of **5** (135 mg, 0.4 mmol) in anh. CH_2Cl_2 (10 ml) at r.t. The mixture was stirred at

reflux for 90 min (TLC control). The mixture was then washed with sat. aq. $NaHCO_3$ soln. (3×20 ml), and the combined aq. layer was re-extracted with CH_2Cl_2. The org. layers were combined, dried, and concentrated under reduced pressure to yield a crude product (165 mg), which was separated by CC (SiO_2), eluting with 35% AcOEt in hexane, to yield **7** (50 mg) and **8** (20 mg).

(1S,2S,7R,10α,12R,17R)-2-Acetoxy-7,17:12,17:15,16-triepoxy-1-hydroxycleroda-3,13(16),14-trien-18,19-olid (**7**). Colorless, amorphous solid. IR (KBr): 2921, 1741, 1728, 1374, 1247, 1201, 1059, 1026. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. EI-MS: 416.2 (8, M^+), 356.1 (14), 275.1 (20), 95.0 (55), 94.0 (50), 81.0 (50), 69.1 (24), 55.0 (36), 43 (100). CI-MS (NH_3): 434.2 (100, $[M+NH_4]^+$). HR-CI-MS: 434.1816 ($[M+NH_4]^+$, $C_{22}H_{28}NO_8^+$, calc. 434.1815).

(1R,2R,7R,10α,12R,17R)-1-Acetoxy-7,17:12,17:15,16-triepoxy-2-hydroxycleroda-3,13(16),14-trien-18,19-olid (**8**). Colorless, amorphous solid. IR (KBr): 2919, 1746, 1744, 1376, 1232, 1053, 1016. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. EI-MS: 416.2 (8, M^+), 368.1 (5), 356.1 (5), 340.1 (6), 105.1 (71), 95.0 (56), 81.1 (50), 69.1 (46), 55.1 (56), 43 (100). HR-ESI-MS: 439.1372 ($[M+Na]^+$, $C_{22}H_{24}NaO_8^+$; calc. 439.1369).

Biological Studies. Compounds **2**–**8** were examined for their receptor-binding affinities in a competitive binding assay against the non-selective antagonist [^{3}H]diprenorphine in membrane preparations derived from *C6μ*, *C6δ*, and *CHOκ* cells.

Briefly, *C6* glioma cells, stably transfected with the μ- or δ-opioid receptors (*C6μ* and *C6δ*) [17], and Chinese hamster ovary cells expressing the human κ-opioid receptor (*CHOκ* cells) [18], were cultured under a 5% CO_2 atmosphere in *Dulbecco*'s modified Eagle medium (for the *C6* cells), or in *Dulbecco*'s modified Eagle medium/nutrient mix *F-12* (for Chinese hamster ovary cells), supplemented with 10% fetal calf serum (FCS). For subculture, one flask from each passage was grown in the presence of geneticin. The cells used for the experiments were grown in the absence of geneticin, with no significant reduction in receptor number. Once the cells had reached confluency, they were harvested in 20 mM *HEPES*-buffered saline (pH 7.4), containing 1 mM EDTA, dispersed by agitation, and collected by centrifugation at 1,600 r.p.m. The cell pellet was suspended in 50 mM *Tris*·HCl buffer (pH 7.4), and homogenized with a *Tissue-Tearor* (*Biospec Products*, Bartlesville, OK, USA). The resultant homogenate was centrifuged for 15 min at 18,000 r.p.m. at a temp. of 4°. The pellet was collected, re-suspended in 50 mM *Tris*·HCl buffer (pH 7.4), separated into 0.5 ml aliquots (0.75–1.0 mg protein), and frozen at −80°. Protein concentrations were determined by the method of *Lowry et al.* [19], using bovine serum albumin (BSA) as standard.

For ligand binding, the cell membranes (30–60 μg protein) were incubated at 25° in 50 mM *Tris*·HCl buffer (pH 7.4) for 1 h with 0.2 nM [^{3}H]diprenorphine (final volume of 1 ml). Non-specific binding was defined with 10 μM naloxone. Reactions were terminated by filtration through glass-fiber filters (*Schleicher & Schuell*, Keene, NH, USA), and mounted in a *Bandel* 24-well harvester. The filters were washed three times with ice-cold *Tris*·HCl (pH 7.4), and the radioactivity retained was determined by scintillation counting after the addition of *Ultima Gold* liquid-scintillation fluid (3 ml).

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