Full Paper

Synthesis and Characterization of Thiazolo- and Thiazinomorphinans and Their Intermediate Products as Novel Opioid-Active Derivatives*

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A new procedure was elaborated for the synthesis of potentially opioid-active thiazolo- and thiazinomorphinans. These derivatives and some intermediates related with the synthesis were tested in opioid receptor binding studies. Two compounds showed remarkable μ opioid activities and specificities. The ligand-stimulated [^{35}S]GTPγ*S* assays confirmed for both compounds the potent full agonist profile at the μ receptor and for the benzothiazinomorphinan derivative also the δ receptor full agonist character. The structures of these remarkably effective compounds were analyzed with the aid of computational chemistry calculations.

Keywords: Heterocycles / Morphinans / Morphine / Opioid activity / Thiazine / Thiazole

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Introduction

The development of potent and selective opioid-active compounds is still one of the important targets of medicinal chemistry. Therapeutic potential and demand of opioid analgesics with reduced undesirable side-effects have initiated vast amounts of scientific efforts, which have resulted in the appearance of a number of new opioid analgesics and significant expansion of knowledge on the opioid pharmacology [1]. Besides analgesia several other indications were also revealed and introduced clinically in connection with the applicability of opioids such as the management of (drug) dependence, depression, constipation, diarrhea, etc. [2]. Several semisynthetic morphinans anullated with new heterorings were found to have high potency and selectivity towards opioid receptors. It has been a commonly accepted theory that the introduction of an appropriate spacer unit between ring C of the morphinan backbone and those moieties important in the interactions with the binding site of the receptor (e.g., guanidinyl moiety in 5′-guanidinonaltrindole, GNTI) leads to a molecule more suitable spatially to bind to opioid receptors [3]. The introduced spacers could be divided into different groups on the basis of their size and chemical character, for example, indole [4a], furan [4b], and pyrrole [4c] moieties. Up to this date the most successful compounds from this research direction are naltrindole (NTI), a highly active and selective δ opioid receptor antagonist and GNTI, an efficient κ opioid receptor antagonist. Both compounds were derived from naltrexone and have been widely used in scientific research as highly selective opioid receptor ligands (Fig. 1) [1].

As a subclass of heteroring-fused morphinans some thiazolo-derivatives were also reported. The first aminothiazole-fused morphinans were prepared by Görlitzer and Schumann. The new heteroring moiety was introduced to the ring C of oxycodone in positions 6 and 7 [5a], then in the same position to hydrocodone and hydromorphone [5b]. Aminothiazole-fused levorphanol, cyclorphanol and their

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*This paper is dedicated to the memory of Professor András Lipták, deceased 11/06/2012

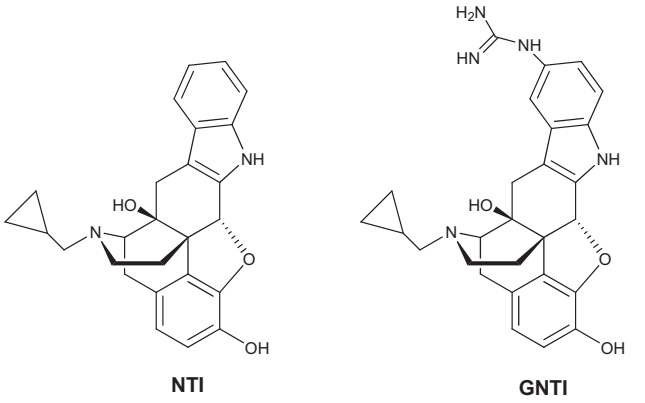
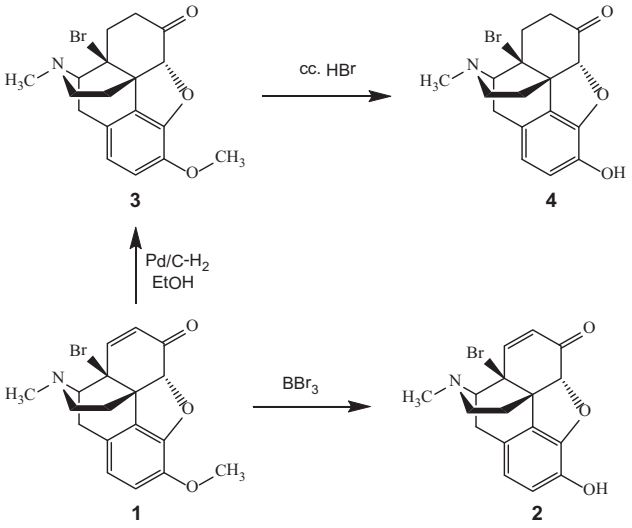


Figure 1. Structure of potent and selective opioid antagonist heteroring-fused morphinans.



Scheme 1. Transformations of 14β-bromocodeinone (**1**).

17-cyclobutylmethyl congeners were found recently to posses very high affinity towards all three opioid receptor subtypes with some κ-selectivity [6]. The 14-hydroxy derivative of the cyclorphanol-derived aminothiazolomorphinan exhibited high μ-κ affinity and full μ-agonistic properties [7]. We previously reported on a new strategy for the formation of morphinans with new S- and N-containing heterorings fused to ring C [8] exploiting the observation that 14β-bromocodeinone can react as an α-haloketone. Here we present the extension of these methodologies for the preparation and study of pharmacologically more interesting 3-hydroxy congeners. The synthetic procedure involved also the preparation of 14β-bromo derivatives of 6-ketomorphinans, some of which were intermediates of the aimed heteroring-fused morphinans, to expand further the existing structure–activity relationships for 14β-substituted morphinans.

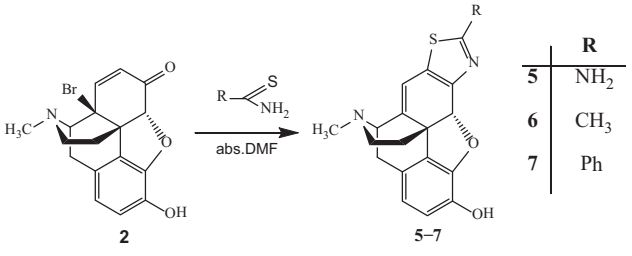
Results and discussion

The synthesis of 6,7-thiazolomorphinans with the 3-hydroxy motif was previously achieved by direct 3-*O*-demethylation of 3-*O*-methyl intermediates [8a]. This L-selectride-mediated selective ether cleavage [9] was performed for 14 days, resulting in 3-deprotected morphinans in average yields. The reason for the use of L-selectride is the known high sensitivity of 6,8-morphinandienes to acidic conditions [8b, 10]. However, this methodology has some drawbacks such as the relatively low yield and the very long reaction time. Therefore it was decided to circumvent the *O*-demethylation step of the morphinandienes and perform the construction of 1,3-thiazole and 1,4-thiazine rings from 14β-bromomorphinone (**2**).

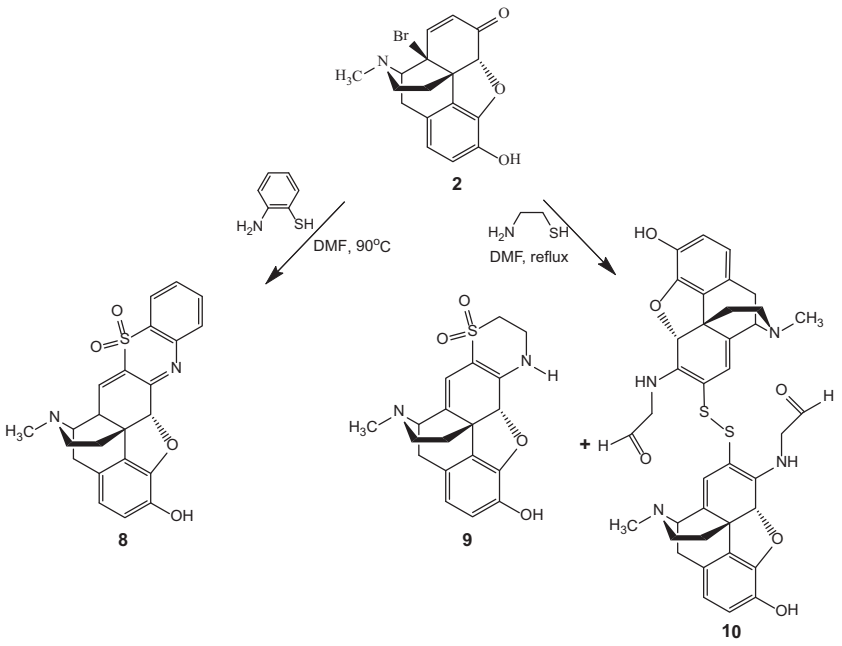
The synthesis of 14β-bromomorphinone (**2**) (Scheme 1) was reported by Osei-Gyimah and Archer [11]. 14β-Bromocodeinone (**1**), a long-known 6-ketomorphinan [12], was *O*-demethylated under relatively mild conditions using boron tribromide solution. The analgesic activity of derivative **2** was tested on guinea pig ileum and found half as potent as the reference compound normorphine [11].

Compound **2** was synthesized in 76% yield. It was recognized that saturated dihydrocodeinone derivatives in the 14β-bromo-series have not been reported up to this work. Therefore we prepared 14β-bromodihydrocodeinone (**3**) from compound **1** with the usual catalytic hydrogenation over 10% Pd/C catalyst in excellent yield (92%). The deprotection of compound **3** was accomplished with refluxing concentrated HBr solution giving rise to 14β-bromodihydromorphinone (**4**) ready for pharmacological studies.

After having the proposed new starting material in hand, 14β-bromomorphinone (**2**), the same protocol was applied for the formation of 6,7-thiazolomorphinans as it was published previously [8a]. We speculated that the 3-*O*-demethylation of the reacting 6-ketomorphinan will not affect the reactivity of ring C. In accordance with our expectations, the synthesis of 2′-substituted thiazolomorphinans **5–7** was achieved in acceptable (45–56%) yields (Scheme 2).



Scheme 2. Synthesis of 3-hydroxy thiazolomorphinans **5–7**.



Scheme 3. Reaction of 14β-bromomorphinone (**2**) with β-aminothiols.

After the successful adaptation of a modified Hantzsch-type thiazole synthesis for the preparation of 3-unprotected morphinans **5–7**, we aimed to repeat our thiazine formation reactions also starting from 14β-bromomorphinone (**2**) [8b]. The reaction was carried out in the presence of 2-aminothiophenol, resulting in benzothiazinomorphinan **8** having the cyclic sulfur in the highly oxidized sulfone form. This phenomenon was in line with our previous observation, most probably as a result of an *in situ* photooxidation mechanism (Scheme 3) [8b].

The reaction of cysteamine with compound **2** gave rise to two new morphinans **9**, **10** isolated with column chromatography in low yields. The first eluted component of the resulting mixture was a dihydrothiazinomorphinanediene **9**. The spectroscopic studies revealed that the cyclic sulfur was again in the oxidized sulfone form due to similar reasons. After isolating the second component, the analytical characterization confirmed that this derivative is a bis-morphinan **10** attached through the disulfide segment arisen from two opened 1,4-thiazine rings. The formation of this unusual product was the consequence of the occurrence of an α-hydroperoxy sulfide-type photooxidation intermediate [8b].

Activity and selectivity of new analogs **1–10** towards μ, δ, and κ opioid receptors were evaluated by radioligand binding assays according to the known procedures [13]. Rat brain membranes were used as a source of μ and δ receptors, and guinea pig brain membranes as a source of κ receptors. K_i values at μ, δ, and κ opioid receptors, determined against [^{3}H]DAMGO, [^{3}H][Ile^{5,6}]deltorphin-2 and [^{3}H]U69,593, respectively, were collected in Table 1.

For comparison, opioid binding data for morphine were also included. Within the group of 14β-bromo derivatives **1–4** the general structure–activity relationships of the morphinan-6-ones can be followed up. The methyl ether protection of the 3-hydroxyl function generally results in decreased opioid activity. The saturation of the Δ[7, 8] bond leads to increased affinity which is clear in comparisons of compounds **1** *versus* **3** or **2** *versus* **4**. These two factors point to a superior activity in 14β-bromodihydromorphinone (**4**) which is in line with our data. Compound **4** was found to posses two to three times increased affinity compared to morphine towards each opioid receptor maintaining the remarkable μ-selectivity.

In connection with the opioid activity of 2′-substituted thiazolomorphinans **5–7** we can conclude that the presence of the strong H-bond donor and acceptor amino-group at position 2′ was unfavorable especially at δ and κ receptors. The methyl and phenyl substitution of this amino function gradually increased the affinities, most characteristically towards μ-receptors, however the binding activity of compound **7** was still weaker than that of the reference compound morphine.

In the opioid binding studies for the three products obtained from reactions with β-aminothiols, **8–10**, significant differences were observed. Benzothiazinomorphinan **8** was found to posses approximately three times higher activity towards μ- and κ-receptors and 20 times higher affinity towards δ receptors in comparison to the data of morphine. For this compound the μ-selectivity was less dominant. On the other hand derivatives **9** and **10** were found to be fully inactive towards all receptor subtypes.

Table 1. *In vitro* opioid receptor binding affinities and selectivities in rodent brain

Compound	K_i (nM)			Selectivity	
	μ-Receptor[a)]	δ-Receptor[b)]	κ-Receptor[c)]	δ/μ	κ/μ
Morphine[d)]	6.55 ± 0.74	217 ± 19	113 ± 9	33	17
1	1642 ± 368	571 ± 89	>10 000	0.3	>6
2	64.2 ± 3.79	765 ± 1	1070 ± 158	12	17
3	29.4 ± 8.26	437 ± 55	864 ± 41	15	29
4	2.50 ± 0.34	81.6 ± 17.4	68.2 ± 11.9	33	27
5	75.3 ± 13.4	>10 000	1241 ± 190	>133	16
6	77.4 ± 12.1	72.1 ± 10.8	848 ± 56	0.9	11
7	15.9 ± 2.87	95.1 ± 30.1	140 ± 15	6.0	8.8
8	2.23 ± 0.29	8.60 ± 0.68	29.4 ± 5.63	3.9	13
9	3491 ± 284	1285 ± 385	>10 000	0.4	>2.9
10	>10 000	>10 000	>10 000	–	–

All values are expressed as mean ± SEM of three determinations performed in duplicate.
a) Determined against [^{3}H]DAMGO.
b) Determined against [^{3}H][Ile^{5,6}]deltorphin-2.
c) Determined against [^{3}H]U69,593.
d) Taken from ref. [14].

Table 2. *In vitro* agonist potencies in CHO cells expressing human opioid receptors[a)]

Compound	μ-Receptor		δ-Receptor		κ-Receptor	
	EC_{50} (nM)	% Stim[b)]	EC_{50} (nM)	% Stim[c)]	EC_{50} (nM)	% Stim[d)]
Morphine	39.0 ± 9.9	102 ± 5	617 ± 69	116 ± 21	1475 ± 760	75.5 ± 2.9
4	17.0 ± 8.6	124 ± 11	169 ± 90	84.0 ± 4.3	234 ± 42	44.2 ± 8.2
8	12.6 ± 4.4	113 ± 7	13.2 ± 1.7	101 ± 1	225 ± 2	87.7 ± 8.7

a) Membranes from CHO cells stably transfected with human μ, δ, or κ opioid receptors were incubated with varying concentrations of the compounds.
b) Compared to DAMGO.
c) Compared to DPDPE.
d) Compared to U69,593. Data represent mean ± SEM.

After establishing the binding affinities of new morphinans, binding affinities of compounds **4** and **8** were further examined by *in vitro* competition binding assays using rodent brain membranes (Table 2).

In these ligand-stimulated [^{35}S]GTPγS binding studies we used membranes from Chinese hamster ovary (CHO) cells stably transfected with human opioid receptors [14]. This method is appropriate for the characterization of the agonistic profile of targeted derivatives. These tests confirmed for both compounds **4** and **8** that they were potent full agonists at the μ receptor, and benzothiazinomorphinan derivative **8** also at the δ receptor.

In analyzing the structure of the most potent compounds, it can be concluded that 14β-bromo substitution at the morphinan backbone could effectively replace the well-known 14β-hydroxyl function (as in case of oxymorphone) or 14β-methoxy moiety (as in case of 14-methyloxymorphone) without losing significant μ activity and selectivity. For the evaluation of the activity of compound **8**, the density functional theory (DFT)-optimized structure was calculated and compared to the structure of NTI (Fig. 2).

The superimposition of the structures of compound **8** and NTI was generated by overlaying the A rings of the geometry optimized structures. Besides the 3-hydroxyl functions the positions of the tertiary amino moieties were found in good agreement and the whole morphinan skeletons showed similar conformations. Of course, there are two important structural differences of the backbone (*N*-cyclopropylmethyl *vs*. *N*-methyl and the presence of 14-OH in NTI) which could definitely affect the opioid activity. However, the main differences arise from the heteroring moieties annulated to ring C. The nitrogen atoms of the fused heterorings can be found at the same position and the second homoaromatic rings anellated to the heterorings are also in similar positions. The

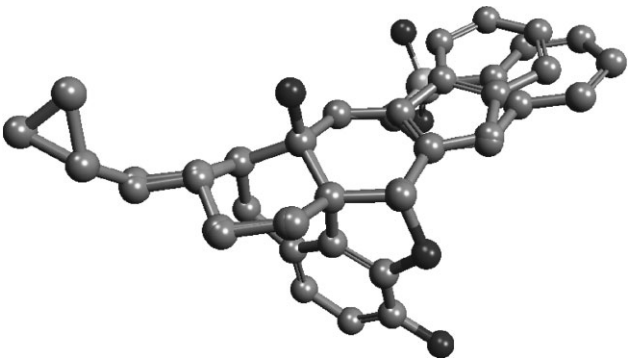


Figure 2. Superimposition of the DFT-optimized structures of compound **8** and NTI.

presence of the sulfone moiety represents significant differences due to electronegativity and H-bond acceptor abilities. Most probably this factor is responsible for the considerable difference in the opioid activities of the selective δ receptor ligand NTI and our μ/δ receptor full agonist benzothiazinomorphinan **8**.

The comparison of the DFT-optimized structures of 14β-bromodihydromorphinone (**4**) and its 14β-methoxy congener showed not surprisingly a high degree of conformity with regard to the positions of most important moieties of the morphinan backbone. However, the calculated Gasteiger–Hückel charges projected on the solvent-accessible surfaces confirmed some interesting differences in terms of the charge-density in the proximity of position 14. In case of the extremely potent μ-agonist 14β-methyloxymorphone the O-atom of the methoxy moiety represents a characteristic negatively charged molecular segment highly shielded by the methyl substituent. This molecular architecture might be responsible for H-bond acceptor functions. On the contrary, position 14 of compound **4** is derivatized with a less negatively charged Br-atom with limited electronic capabilities for the formation of H-bonding, even though there is no structural limitation for accessing this area of the molecule. These results might underline the influence of substituents in the region of position 14 in agreement with the general findings of Ferguson and coworkers [15].

It is clear from the previously presented results that the precise position of a benzoheterocyclic moiety attached to the ring C is less important for achieving remarkable activity and subtype selectivity at opioid receptors than the presence and position of electron withdrawing groups (e.g., sulfone) in this part of the molecule. It can be also concluded that the proper electronic character and ability for H-bond formation of the substituent in position 14 considerably affects the opioid activity.

Conclusions

Our previous procedures for the synthesis of thiazolo- and thiazinomorphinans were extended for the preparation of pharmacologically more promising 3-hydroxyl derivatives. These derivatives and some intermediates related with the synthesis were tested in opioid receptor binding studies. The two most potent compounds were further examined in ligand-stimulated [^{35}S]GTPγS assays, and it was found that both compounds **4** and **8** were potent full agonists at the μ receptor, and the benzothiazinomorphinan derivative **8** also at the δ receptor. The structures of these remarkably effective compounds were analyzed with the aid of computational chemistry calculations and considerable structure–activity conclusions were drawn.

Experimental

General information

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Thin layer chromatography was performed on precoated Merck 5554 Kieselgel 60 F_{254} foils using dichloromethane/methanol = 8:2 as mobile phase; the spots were visualized with Dragendorff's reagent. ^{1}H NMR spectra were recorded on a Varian Gemini 200 spectrometer (200 MHz), chemical shifts are reported for unambiguously identifiable signs in ppm (δ) from internal TMS, and coupling constants (*J*) are measured in Hz. Mass spectra were recorded on a Varian MAT 44 S apparatus. IR spectra were recorded with a Mattson Galaxy Series FTIR 3000 spectrometer (in cm^{-1}). Elemental analyses (C, H) were obtained on a Carlo Erba 1108 analyzer.

14β-Bromomorphinone (2)

This compound was synthesized according to the procedure of Osei-Gyimah and Archer [11] in 66% yield. The physical and spectral properties of our product were in agreement with previously reported data.

*14β-Bromodihydrocodeinone (**3**)*

A mixture of 375 mg (1 mmol) of the unsaturated codeinone **2**, 90 mg of Pd/C (10%), and 200 mL of ethanol (96%) was hydrogenated at 35 psi (r.t.) for 10 h. The obtained mixture was filtered through a short pad of Celite and washed with 3 × 10 mL of dichloromethane/methanol = 8:2. The resulting organic solution was concentrated to dryness under vacuum. The crude compound was found pure enough to step forward without purification.

Off-white solid; m.p.: 155°C (dec.); yield: 347 mg (92%); calculated for $C_{18}H_{20}BrNO_3$: C, 57.15; H, 5.33%; found: C, 57.07; H, 5.41%; ν_{max}(KBr disc) 1724; MS *m/z* (%) 379 (M^++1, 100); δ_H (200 MHz, DMSO-d_6) 6.67 (1H, d, H1, $J_{1,2}$ = 7.9), 6.62 (1H, d, H2, $J_{1,2}$ = 8.0), 4.67 (1H, s, $H5_a$), 3.87 (3H, s, OCH_3), 2.42 (3H, s, N–CH_3).

*14β-Bromodihydromorphinone (**4**)*

A mixture of 378 mg (1 mmol) dihydrocodeinone **3** and 2.5 mL of 48% HBr solution was heated at 95°C for 1.5 h. The resulting

mixture was poured into 100 mL of saturated $NaHCO_3$ solution (pH ~8) and extracted with EtOAc (3 × 25 mL). The organic phase was washed with water and brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the off-white foam was subjected to column chromatography (eluent: dichloromethane/methanol 8:2).

Off-white solid; m.p.: >250°C; yield: 229 mg (63%); calculated for $C_{17}H_{18}BrNO_3$: C, 56.06; H, 4.98%; found: C, 56.01; H, 5.09%; ν_{max}(KBr disc) 3392, 1731; MS *m/z* (%) 365 ($M^+ + 1$, 90); δ_H (200 MHz, DMSO-d_6) 8.9 (1H, br s, OH), 6.71 (1H, d, H1, $J_{1,2} = 8.1$), 6.66 (1H, d, H2, $J_{1,2} = 8.1$), 4.66 (1H, s, $H5_a$), 2.38 (3H, s, N–CH_3).

General procedure for the synthesis of thiazolomorphinanedienes

The mixture of **2** (1000 mg, 2.64 mmol) and thioamide (2.64 mmol) was dissolved, and then heated to reflux in abs. DMF (10 mL) for 30 min. The mixture was diluted with water (10 mL), the pH of it was adjusted to 8 by the dropwise addition of concentrated ammonia solution and then extracted with ethyl acetate (3 × 20 mL). The organic phase was separated; the solvent was removed *in vacuo*. Crystalline product was precipitated by addition of anhydrous methanol and purified by means of column chromatography (dichloromethane/methanol 8:2). Compounds **5–7** were obtained in this route in 45, 54, and 56% yields, respectively.

The physical and spectral properties of products **5–7** were in agreement with previously reported data [8a].

General procedure for the formation of 1,4-thiazine or benzo-1,4-thiazine rings on the morphinan skeleton

A mixture of **2** (1000 mg, 2.64 mmol) and β-aminothiol (2.64 mmol) was dissolved and heated to reflux in anhydrous DMF (10 mL) for 30 min. The mixture was diluted with water (10 mL), the pH was adjusted to 8 by the dropwise addition of concentrated ammonium hydroxide solution. The emulsion was extracted with ethyl acetate (3 × 20 mL). The organic phases were combined; the solvent was removed *in vacuo*. A crystalline product was precipitated by the addition of abs. methanol.

*6,7:5′,6′-(2′,3′-Benzo-1,4-thiazine-1′,1′-dioxide)-7,8-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan (**8**)*

Raw product was purified by means of column chromatography (dichloromethane/methanol 8:2). Yellow solid; mp.: >250°C; yield: 466 mg (42%); calculated for $C_{23}H_{20}N_2O_4S$: C, 65.70; H, 4.79%; found: C, 65.56; H, 4.91%; ν_{max}(KBr disc) 3342, 1385, 1447, 1180; MS *m/z* (%) 421 (M^++1, 100); δ_H (200 MHz, DMSO-d_6) 9.97 (1H, br s, OH), 7.30–7.01 (4H, m, Ar), 6.66 (2H, 2d, H1, H2, J_{1-2} 8.3), 5.66 (1H, d, H8, J_{8-14} 6.1), 4.21 (1H, s, H5), 2.54 (3H, s, NCH_3).

*6,7:5′,6′-(2′,3′-Dihydro-1,4-thiazine-1′,1′-dioxide)-6,7-didehydro-8,14-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan (**9**)*

Compounds **9** and **10** were separated by means of column chromatography (dichloromethane/methanol/concentrated ammonium hydroxide = 90:9:1). Compound **9** was the first eluted component. Pale yellow solid; mp.: >250°C; yield: 187 mg (19%); calculated for $C_{19}H_{20}N_2O_4S$: C, 61.27; H, 5.41%; found: C, 61.35; H, 5.55%; ν_{max}(KBr disc) 3351, 2980, 1364, 1230, 1169; MS *m/z* (%) 373 ($M^+ + 1$, 59); δ_H (200 MHz, DMSO-d_6) 9.85 (1H, br s, OH), 8.56 (1H, br s, NH), 6.59 (2H, 2d, H1, H2, J_{1-2} 8.0), 5.87 (1H, s, H8), 4.21 (1H, s, H5), 2.57 (3H, s, NCH_3).

Bis-[6-(1′-amino-ethan-2′-al)-6,7-didehydro-8,14-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan-7-yl]disulfide (10)

Compound **10** was the second eluted component. Grey solid; mp.: 212°C (dec.); yield: 262 mg (14%); calculated for $C_{38}H_{38}N_4O_6S_2$: C, 64.20; H, 5.39%; found: C, 64.35; H, 5.48%; ν_{max}(KBr disc) 3349, 2991, 2804, 1727, 1241, 1160; MS *m/z* (%) 711 (M^++1, 29), 356 [$(M/2)^+$+1, 100]; δ_H (200 MHz DMSO-d_6) 10.25 (2H, br s, 2 OH), 9.31 (2H, t, 2 CHO, *J* 2.8), 6.65 (4H, 2d, H1, H2, J_{1-2} 8.0, H1′, H2′, $J_{1'-2'}$ 8.0), 5.62 (2H, s, H8, H8′), 4.41 (2H, s, H5, H5′), 3.69 (4H, d, NH–CH_2–CHO, *J* 3.0), 2.47 (6H, s, 2 NCH_3).

Pharmacological experiments

Materials

Opioid radioligands, [^{3}H][d-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO), [^{3}H]5α,7α,8β-(−)*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide ([^{3}H]U69,593) and radiolabeled guanosine-5-*O*′-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) were purchased from PerkinElmer (Boston, MA, USA). [^{3}H][Ile^{5,6}]deltorphin II was obtained from the Institute of Isotopes Co. Ltd. (Budapest, Hungary). Naloxone hydrochloride (NX), Tris, unlabeled GTPγS, guanosine diphosphate (GDP), DAMGO, d-Phe–Cys–Tyr–d–Trp–Arg–Thr–Pen–Thr–NH2 (CTAP), nor-binaltorphimine (nor-BNI), and EGTA were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). Morphine hydrochloride was obtained from Gatt-Koller GmbH (Innsbruck, Austria).

All other chemicals were of analytical grade and obtained from standard commercial sources.

Brain membrane preparations

Membranes were prepared from Sprague–Dawley rat or guinea pig brains. Brains without cerebella were homogenized on ice in 5 v/w of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and diluted in 30 v/w of the same buffer. After centrifugation at 40 000×*g* for 20 min at 4°C, the pellets were resuspended in 30 v/w of 50 mM Tris–HCl buffer (pH 7.4) and incubated at 37°C for 30 min. The centrifugation step described above was repeated, the final pellets were resuspended in 5 v/w of 50 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at −80°C until use. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard.

Opioid receptor binding assays

Binding experiments were performed in 50 mM Tris–HCl buffer (pH 7.4.) in a final volume of 1 mL containing 0.3–0.5 mg protein and at least 10 concentrations of test compound. Rat brain membranes were incubated either with [^{3}H]DAMGO (1 nM, 45 min, 35°C) or [^{3}H][Ile^{5,6}]deltorphin II (0.5 nM, 45 min, 35°C). Guinea pig brain preparations were incubated with [^{3}H]U69,593 (1 nM, 30 min, 30°C). Reactions were terminated by rapid filtration through Whatman GF glass fiber filters type GF/B pre-soaked in 0.1% polyethylenimine for 1 h at 4°C for [^{3}H]U69,593, or type GF/C for [^{3}H]DAMGO and [^{3}H][Ile^{5,6}]deltorphin II) using a Brandel M24R Cell Harvester (Gaithersburg, MD, USA). Filters were washed three times with

5 mL of ice-cold 50 mM Tris–HCl buffer (pH 7.4). Non-specific binding was determined in the presence of 10 μM naloxone. The bound radioactivity was measured by liquid scintillation counting using a Beckman CoulterTM LS6500 (Beckman Coulter Inc., Fullerton, CA, USA).

Inhibition constant (K_i) values were calculated from competition binding curves using the nonlinear least-square curve fitting by GraphPad Prism software (v3; GraphPad Software Inc., San Diego, CA, USA). All experiments were performed in duplicate and repeated two to six times.

[^{35}S]GTPγS (guanosine-5′-O-(3-[^{35}S]thio)-triphosphate) binding assay

Rat brain membranes (10 μg of protein) were incubated for 60 min at 30°C in Tris–EGTA buffer (50 mM Tris–HCl buffer, 3 mM $MgCl_2$, 1 mM EGTA, 100 nM NaCl, pH 7.4) containing 30 μM GDP, 0.05 nM [^{35}S]GTPγS and appropriate concentrations of test compound in a final volume of 1 mL. Non-specific binding was measured in the presence of 100 μM unlabeled GTPγS. Reactions were terminated by vacuum filtration through Whatman GF/B glass fiber filters and bound [^{35}S]GTPγS retained on the filters was determined as described for opioid receptor binding assays. Stimulation of [^{35}S]GTPγS binding produced by the test compound is given as percentage of the basal activity (defined as 100%, measured in the absence of test compound). The EC_{50} (nM, concentration of ligand to elicit half-maximal effect) and E_{max} (%, maximum stimulation) were calculated using nonlinear regression analysis and sigmoidal curve fitting with the GraphPad Prism software. All experiments were performed in triplicate and repeated at least three times.

Computational procedure

We carried out the geometry optimization at Becke's three parameter hybrid (B3LYP) [16] levels in the DFT with the basis set 6–31G(d,p) using Gaussian 03 [17]. The solvent effect was not considered.

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