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PREPARATION OF AN AFFINITY GEL FOR OPIATE RECEPTORS

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[D-Ala², Leu⁵]Enkephalin was coupled with aminohexyl-Sepharose at its C-terminal. This gel could bind solubilized opiate receptors prepared from rat brain using digitonin. This suggests that the prepared gel is a prominent candidate as an affinity gel for opiate receptors.

KEYWORDS — enkephalin derivative; peptide synthesis; affinity gel; opiate receptor; radioreceptor binding assay

It has been well established that there are multiple opiate receptors,¹⁾ but it is still unclear whether they represent different molecular species or are different conformation of a single receptor. Isolation and characterization of the receptors is of prime importance. Our earlier study showed the usefulness of photoaffinity labeling of opiate receptors to identify them,²⁾ but this causes them to lose the ability to bind to opiates. Therefore, we decided to use solubilization and partial purification of opiate receptors which have been reported by several laboratories.³⁾ Recently, attempts to purify opiate receptors by affinity chromatography was reported from three laboratories.⁴⁾ In our present study, we prepared an affinity gel which has an enkephalin derivative as an affinity ligand to the receptors.

Enkephalin analogs with single substitution have the following characteristics: an intact L-Tyr¹ moiety is vital for activity; substitution of D-amino acids at position 2, in particular D-Ala, leads to increased activity; a wide variety of manipulations are possible at C-terminal without destroying activity.⁵⁾ Therefore, we chose [D-Ala², Leu⁵]enkephalin (DALE)⁶⁾ as an affinity ligand for opiate receptors, which was then attached to a gel at its C-terminal.

We used trifluoroacetyl (CF₃CO) group as the N^α-blocking group of the final enkephalin derivative which was coupled with a gel because the CF₃CO-group was readily removed by alkali treatment.⁷⁾ However, the CF₃CO-group has found limited use for the protection of the α-amino group, mainly because of possible racemization during coupling.⁸⁾ Therefore, the Boc-group was used as the N^α-blocking group during coupling. Then the Boc-group of the resulting peptide was removed and the CF₃CO-group was introduced to the N^α-position of the peptide, which was then coupled to a gel at its C-terminal.

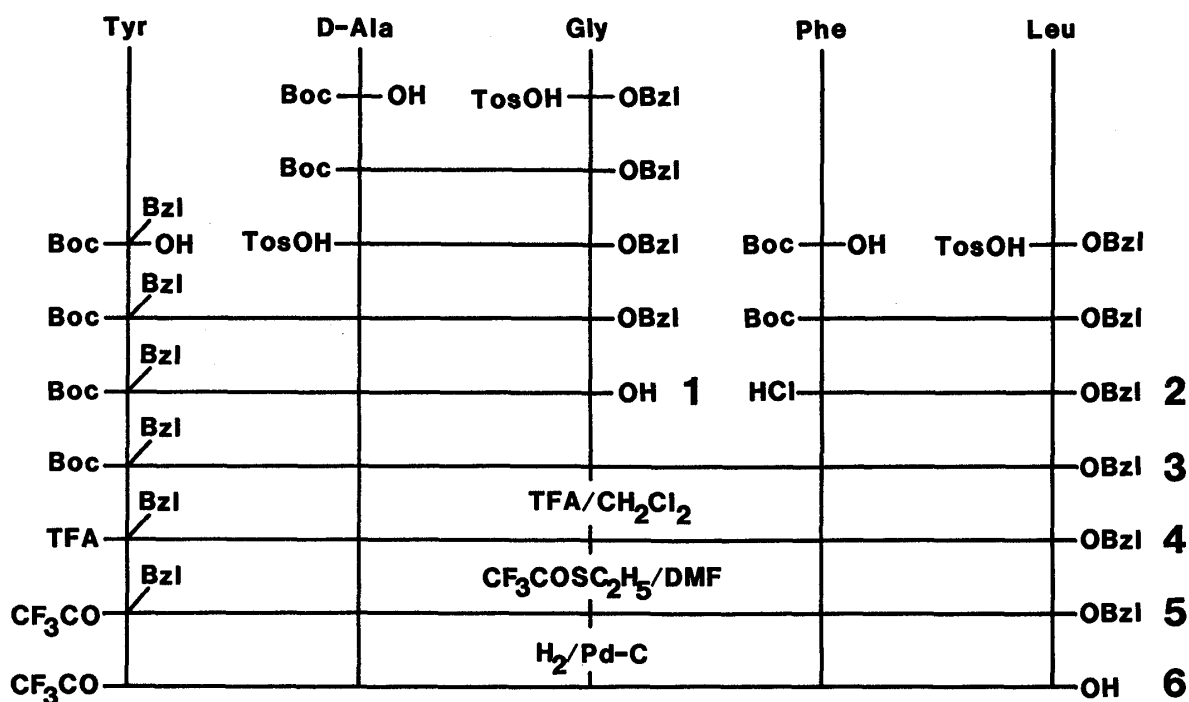


Chart 1. A Synthesis Route for CF₃CO-[D-Ala², Leu⁵]Enkephalin

Enkephalin derivatives were synthesized by conventional solution methods and the synthesis route is shown in Chart 1. A general procedure is as follows: N^α-blocked amino acid or peptide (1 mmol) in THF was cooled at -15°C and 4-methylmorpholine (1 mmol) and isobutyl chlorocarbonate (1 mmol) were added to the solution; the mixture was stirred under cooling for 7 min and then an ester of amino acid or peptide (1 mmol) as a salt and 4-methylmorpholine (1 mmol) in THF was added to the mixture; the resulting mixture was stirred for 2 h under cooling, then at r.t. overnight; the reaction mixture was post-treated in the usual way.⁹⁾ Boc-Tyr(Bzl)-D-Ala-Gly (1), which was characterized as a dicyclohexylamine salt [mp 159-163°C], was coupled with Phe-Leu-OBzl HCl (2) [mp 162-164°C]. Recrystallization of the product from AcOEt/pet. ether gave Boc-Tyr(Bzl)-D-Ala-Gly-Phe-Leu-OBzl (3) [mp 134-138°C; [α]_D -15.3°(c 1.05, DMF) (lit.¹⁰⁾ +11°(c 1, MeOH)); Rf 0.64(A)¹¹⁾; Anal. Calcd for C₄₈H₅₉N₅O₉: C, 67.82; H, 7.00; N, 8.24. Found: C, 67.63; H, 7.15; N, 8.13] in 85% yield. Compound 3 (1 mmol) was treated with TFA (10 ml) in CH₂Cl₂(20 ml) in the presence of anisol (0.4 ml) (0°C, 1 h). Tyr(Bzl)-D-Ala-Gly-Phe-Leu-OBzl TFA (4) was obtained as an amorphous compound and this was used without further purification. S-Ethyl trifluorothioacetate (2 mmol) was treated with compound 4 (1 mmol) in DMF (2 ml) in the presence of triethylamine (2 mmol) and 4-dimethylaminopyridine (r.t., 1 h). Recrystallization of the product from AcOEt gave CF₃CO-Tyr(Bzl)-D-Ala-Gly-Phe-Leu-OBzl (5) [mp 169-170.5°C; [α]_D -10.4°(c 1.01, DMF); Rf 0.60(A); Anal. Calcd for C₄₅H₅₀N₅O₈F₃: C, 63.89; H, 5.96;

N, 8.28. Found: C, 63.77; H, 6.16; N, 8.22] in 92% yield. Catalytic hydrogenation of the compound 5 gave CF₃CO-Tyr-D-Ala-Gly-Phe-Leu (6) quantitatively, which was characterized as a dicyclohexylamine salt [mp 211-216.5°C (decomp.); [α]_D -57.5°(c 0.10, DMF); R_f 0.87(B); Anal. Calcd for C₄₃H₆₁N₆O₈F₃: C, 60.98; H, 7.26; N, 9.92. Found: C, 60.83; H, 7.68; N, 9.95].

Compound 6 was coupled with aminohexyl-Sepharose (AH-Sepharose; Pharmacia) using water-soluble carbodiimide: Compound 6 (0.25 mmol) in DMF (28 ml) was added to the swollen AH-Sepharose (23 ml) in deionized water; to the mixture, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl (5.75 mmol) was added and the pH was adjusted to 4.5; the mixture was gently shaken (r.t., 2 days) and the gel was washed successively with 45% DMF, 0.1 M Tris-0.5 M NaCl (pH 8.0), 0.1 M acetate-0.5 M NaCl (pH 4.0) to remove the unreacted peptide. Then, the gel was shaken in 0.25 M carbonate (pH 10.0)⁷⁾ (r.t., 2 days) to deblock the CF₃CO-group from the peptide coupled to the gel; the gel was washed successively with 0.1 M Tris-0.5 M NaCl (pH 8.0), 0.1 M acetate-0.5 M NaCl (pH 4.0), and deionized water. The gel obtained was used as an affinity gel. Removal of the CF₃CO-group was confirmed qualitatively by detection of di-dansyl-Tyr originating from the free Tyr residue after hydrolysis of the gel by the method of Woods and Wang¹²⁾ (data not shown). Amino acid analysis of the hydrolyzate of the gel with 6 N HCl (110°C, 24 h) showed that ca. 1 μmole of DALE is present in 1 ml of the gel.

The ability of the prepared gel to bind the opiate receptors was tested by radioreceptor binding assay using ³H-[D-Ala², D-Leu⁵]enkephalin (43.6 Ci/mmol; New England Nuclear) as a radiolabeled ligand. Opiate receptors were solubilized from rat brain with digitonin as described by Demoliou-Mason and Barnard.¹³⁾ The prepared gel or an unmodified gel AH-Sepharose was incubated with the solubilized receptors (30°C, 2 h) and the gels were removed by centrifugation (700 x g, 4°C, 5 min). The obtained supernatants were subjected to binding assay. The binding activity of the materials involved in the supernatants were measured by the method of Nakata and Fujisawa.¹⁴⁾ Details of binding assay will be reported elsewhere.¹⁵⁾ Briefly, the assay medium consisted of Mg buffer (10 mM Tes-KOH (pH 7.5) containing 1 mM EGTA-K⁺, 10 mM MgSO₄, 1 mM benzamidine-HCl, 0.01% bacitracin, 0.002% soybean trypsin inhibitor, 1 μM pepstatin, and 0.2 μM phenylmethanesulfonyl fluoride), 0.1% digitonin, 1 mM dithiothreitol, 2 nM radiolabeled ligand, and the obtained supernatants. If the receptors can be bound to the prepared gel, the supernatant obtained from incubation with the gel will contain no opiate receptors and thus it may make a difference in the assay results whether the supernatant used was obtained from incubation with the gel or AH-Sepharose. At 2 nM ³H-ligand, a difference of 230 dpm (specific activity) was measured; at the same condition, except that the solubilized receptors were used directly in the binding assay without pretreatment with gels and non-specific binding was measured in the presence of 10⁻⁵ M DALE, 242 dpm of specific activity was obtained. The agreement between the specific activities shows that the prepared gel could bind opiate receptors specifically.

These results suggest that the prepared gel, AH-Sepharose coupled with DALE, is a prominent candidate as an affinity gel for opiate receptors. In a preliminary experiment, a 450-fold purification of the brain opiate receptors was achieved by using this affinity gel.¹⁵⁾

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- 6) Abbreviations used follow the IUPAC-IUB tentative rules as described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are as follows: DALE, [D-Ala², Leu⁵]enkephalin; TosOH, p-toluenesulfonic acid; TFA, trifluoroacetic acid; Tes, N-Tris[hydroxymethyl]methyl-2-aminomethanesulfonic acid; AH-Sepharose, aminohexyl-Sepharose; THF, tetrahydrofuran; DMF, dimethylformamide.
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- 11) Analytical TLC was performed with silica gel 60-F₂₅₄(Merck) using the following solvent systems: A, CH₂Cl₂-MeOH (10:1); B, AcOEt-pyridine-AcOH-H₂O (60:20:1:11).
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