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CP-70,030 AND CP-75,998 : THE FIRST NON-PEPTIDE ANTAGONISTS OF BOMBESIN AND GASTRIN RELEASING PEPTIDE

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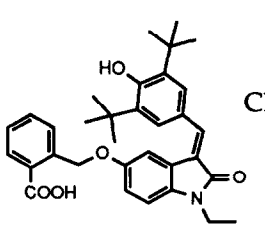
Abstract: CP-70,030 and CP-75,998 were identified in a screening program as compounds able to displace [^{125}I]-gastrin releasing peptide (GRP) from its rat brain receptor. We describe here the syntheses of these compounds and their characterization as bonafide GRP antagonists.

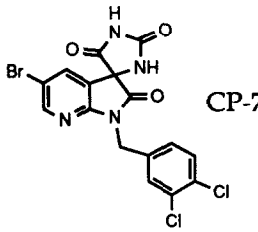
Gastrin Releasing Peptide (GRP) and its closely related amphibian analog bombesin (BN) are autocrine growth factors for some small-cell lung carcinoma (SCLC) cell lines[1,2] and mitogens for Swiss 3T3 cells[3]. GRP antagonists might therefore serve as novel agents for the treatment of SCLC, which accounts for approximately 25% of human lung cancer. The biochemistry of GRP and the therapeutic rationale for antagonists is discussed in several recent articles and reviews[4-8].

In the course of screening to discover non-peptide GRP antagonists, we identified two related compounds (CP-70,030 and CP-75,998) by their ability to displace [^{125}I]-GRP from its rat brain receptor at low micromolar concentrations. In this paper we describe their synthesis, characterization in the binding assay, and verification as antagonists vs. bombesin-stimulated phosphoinositide turnover in rat pituitary GH_3 cells.

GRP val-pro-leu-pro-ala-gly-gly-gly-thr-val-leu-thr-lys-met-tyr-pro-arg-gly-asn-his-trp-ala-val-gly-his-leu-metNH_2
bombesin pyroglu-gln-arg-leu-gly-asn-gln-trp-ala-val-gly-his-leu-metNH_2



CP-70,030



CP-75,998

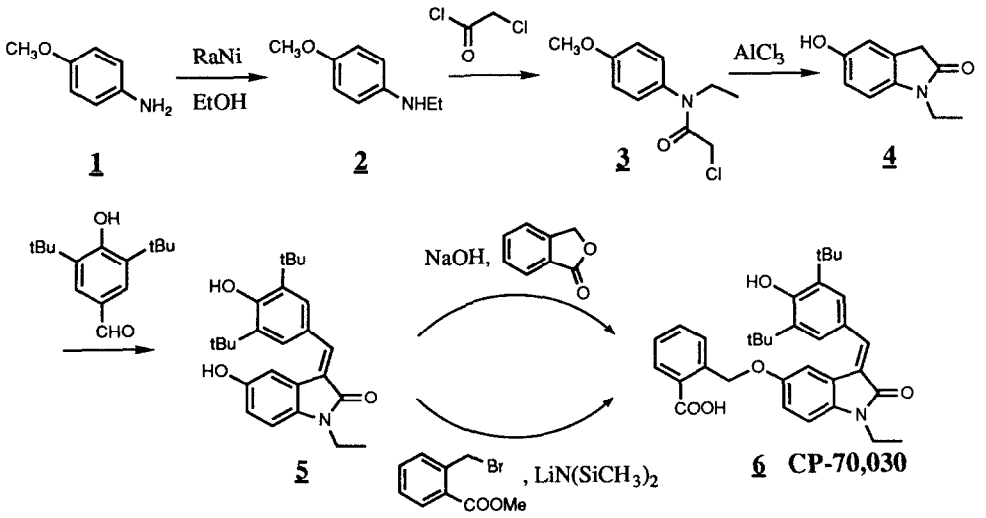
Syntheses

Preparation of CP-70,030 (6) (1-Ethyl-3-[methylene-(3',5'-di-t-butyl-4'-hydroxylphenyl)]-5-(2'-carboxybenzyloxy)oxindole) (Scheme 1):

p-Methoxyaniline 1 was N-alkylated (Agui *et al.*[9])(89% yield), and the product chloroacetylated (79% yield) and cyclized with concomitant O-demethylation (Walker *et al.*[10])(56% yield) to give 4. Condensation of 4 with 3,5-di-t-butyl-4-hydroxybenzaldehyde in methanol/pyrrolidine afforded 5 (67% yield) which was treated with sodium hydoxide followed by phthalide[11] to give 6 (24% yield).

Alternately, **6** was prepared by the reaction of **5** with 2-bromoethylbenzoic acid methyl ester in the presence of lithium bis(trimethylsilyl)amide (67% yield), followed by hydrolysis to give the desired compound **6** (76% yield)(m.p. 180-181°C; m/e 527 (M^+), 392).

CP-70,030, crystallized from ether and dichloromethane, exists in the *E* configuration, with the 3,5-di-t-butylphenol in close contact with the C-4 proton of the indole (Figure 1). However, both thin layer chromatography and HPLC of pure samples show two peaks, suggesting that *E*←→*Z* equilibration occurs in solution. Crystallographic data are available from the authors on request.



Scheme 1

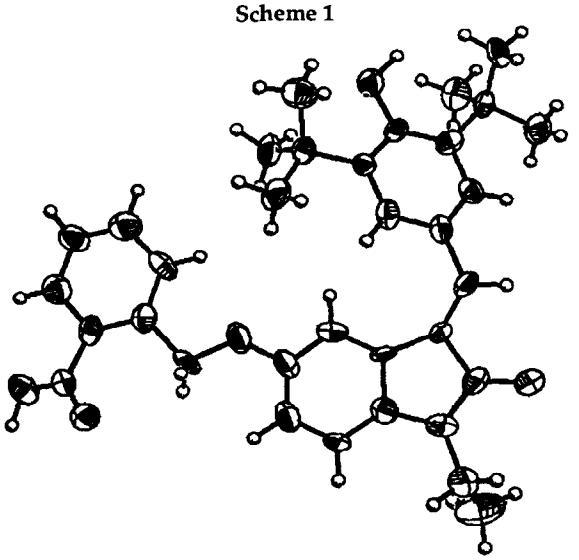
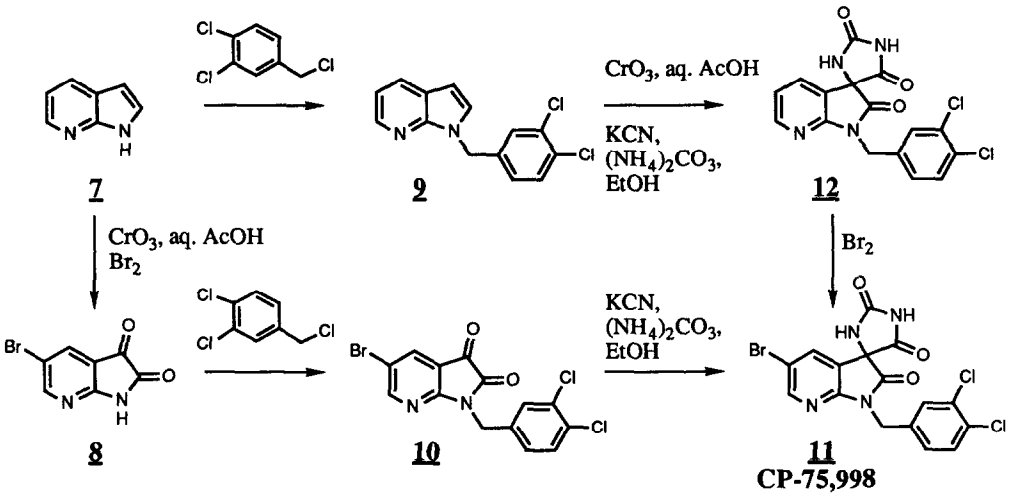


Figure 1 : X-ray Structure of CP-70,030

Preparation of CP-75,998 (11) (1-(3',4'-Dichlorobenzyl)-5-bromo-spiro-[imidazoline-4,3'-azaindoline]-2,2',5-trione) (Scheme 2) :

Azaindole 7 was oxidized with chromium trioxide in aqueous acetic acid[12] and then halogenated to give 8 which subsequently N-alkylated to provide 10. Hydantoin formation by the procedure of Otomaru *et al.*[13] resulted in 11. Alternatively, 7 was N-alkylated[14] with 3,4-dichlorobenzyl chloride to give 9. Chromium trioxide oxidation of 9 followed by spirohydantoin preparation gave 12, which was halogenated[15] to the desired compound 11 (m.p. 221-223(d)°C; m/z 454 (M^+), 456, 458, 259, 224).



Scheme 2

Biological Methods : Ligand Binding

Fresh brain tissue (without pons and medulla) from adult male Sprague-Dawley rats was homogenized (Polytron) in cold 50 mM Tris·HCl buffer, pH 7.4, and diluted to 1 g wet tissue/100 mL. Following centrifugation (30,000g, 15 min), the pellet was resuspended in the same volume of 50 mM Tris.HCl, 100 mM NaCl buffer, pH 7.4, incubated 1 h at 0°, then recentrifuged, and finally resuspended at 0.04 g (original wet tissue weight)/mL 50 mM Tris.HCl buffer, pH 7.4, and kept at 0°C. [^{125}I]-GRP (2000 Ci/mmol, Amersham) was diluted to 0.5 µCi/mL 50 mM Tris.HCl buffer, pH 7.4, 0°C.

Assays were carried out in 96-well microtiter plates, with each well containing 50 µL radioligand, 50 µL test compound in 0.5% v/v DMSO, and 100 µL tissue suspension. Following a 25 min incubation (0-4° with gentle orbital shaking), unbound ligand was removed with a Skatron harvester (0.2% polyethyleneimine presoak, 50 mM Tris pH 7.4, 0° wash) and the bound ligand, on fiberglass mats (Whatman GF/B), was counted in a BetaPlate scintillation counter (LKB). Non-specific binding was estimated by the addition of bombesin (Sigma; 3 µM final concentration) to control wells.

Biological Methods : Functional Characterization

CP-70,030 and CP-75,998 were tested for functional activity vs. bombesin-stimulated inositol phosphate turnover as follows. Rat pituitary GH_3 cells (American Type Culture Collection) were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were labelled with 5 μCi/mL [^{3}H]inositol (American Radiochemical Corporation, St. Louis) for 48 h, at which time the cells were harvested by scraping, centrifugally washed twice in PBS and resuspended in HEPES-buffered Dulbecco's PBS containing 1 mg/mL glucose. The reaction tubes contained 400 μL of this cell suspension plus bombesin (1 - 1000 nM), test compound or vehicle, and 10 mM LiCl in a final volume of 500 μL. After incubation for 30 min, 37°, in a shaking water bath, the reactions were stopped by the addition of 1.5 mL $CHCl_3$:MeOH::1:2, followed by vigorous vortexing and rapid cooling. After addition of 1 mL $CHCl_3$ and 0.5 mL water and vortexing, the phases were separated by centrifugation. The upper (aqueous) layer was collected, heated to 50° for 15 min to remove trace $CHCl_3$, and diluted with 1.5 mL water. A 50% slurry of Dowex 1X8 formate (100-200 mesh, 0.5 mL) was added to adsorb the labelled inositol phosphates. The resin was washed 5 times with 4 mL of 5 mM unlabelled inositol. Finally, the labelled inositol phosphates were eluted with 1 mL of 1.5 M ammonium formate/0.1 M formic acid and counted by liquid scintillation spectrometry. In this system we find 50% maximal stimulation at 2.5 nM bombesin; assays with CP-70,030 and CP-75,998 contained 10 nM bombesin (= 4 x EC_{50}).

Results and Discussion

Both CP-70,030 and CP-75,998 specifically displaced radiolabelled GRP from its rat brain receptors with IC_{50} values of 1.5 - 3 μM. Measurement of bombesin-induced phosphoinositide turnover in rat pituitary GH_3 cells showed that CP-70,030 and CP-75,998 are antagonists with IC_{50} values of 1.5 ± 0.1 μM (Figure 2). In the absence of bombesin, neither compound (up to 30 μM) significantly elevated inositol phosphate production, indicating that they have no measurable agonist activity.

Unfortunately, neither compound (up to 32 μM) displaced labelled GRP from its receptors on the bombesin-responsive human SCLC cell line N592[1]. This considerable species selectivity — at least 30-fold — is comparable to that which we have observed with non-peptide Substance P antagonists[16]. Relatively rigid non-peptide antagonists may thus reveal receptor differences between species that are not apparent using peptides.

Without knowledge of the bioactive conformation of bombesin or GRP — let alone the structure of either peptide bound to the receptor — we cannot speculate about which functional groups of the peptides are mimicked by CP-70,030 and CP-75,998. It is naïve to suppose that the oxindoles map to a tryptophan, or that pendant phenyls map to a phenylalanine or tyrosine. Indeed, though we believe that CP-70,030 and CP-75,998 occupy some of the same physical space at the receptor as the natural ligands, this is not assured by radioligand displacement or functional antagonism.

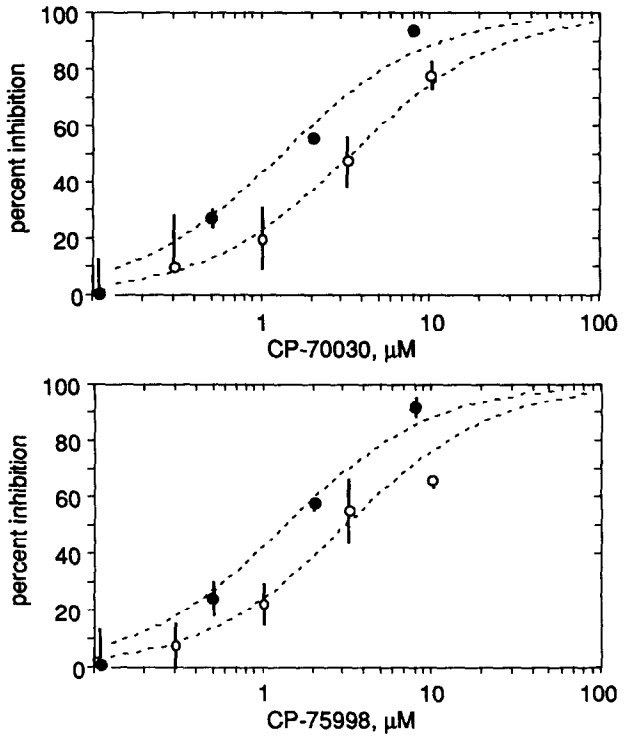


Figure 2 : [^{125}I]-GRP Displacement (○) and Functional Activity (•) of CP-70,030 and CP-75,998
Radioligand binding and phosphoinositide turnover experiments were performed as described in the text.

In conclusion, CP-70,030 and CP-75,998 join the list of non-peptide antagonists of G-protein coupled receptors which have peptide agonists[17]: the Substance P antagonist CP-96,345 we recently described[16], CCK-A and CCK-B antagonists[18], angiotensin II antagonists[19], vasopressin antagonists[20], oxytocin antagonists[21], and C5a antagonists[22].

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