aldehyde, 100-10-7; 3,4,5-trimethoxy-α-1,2-propadienylbenzenemethanol, 127034-56-4; 4-bromo-*N*,*N*-dimethylaniline, 586-77-6; 3,4,5-trimethoxyacetophenone, 1136-86-3; 2,5-dimethoxyacetophenone, 1201-38-3; 2,4,6-trimethoxyacetophenone, 832-58-6; 4-acetamidobenzadlehyde, 122-85-0; 2-methoxyacetophenone, 579-74-8; 3,4-dimethoxyacetophenone, 1131-62-0; 2,4-dimethoxyacetophenone, 829-20-9; 4-hydroxy-3,5-dimethoxyacetophenone, 2478-38-8; 2,3,4-trimethoxypropiophenone, 18060-58-7; 3,4,5-trimethoxypropiophenone, 5658-50-4; 2,5-dimethoxypropiophenone, 5803-30-5; 1-(2,3,4-trimethoxyphenyl)-1-butanone, 108401-78-1; 4-pyridinecarboxaldehyde, 872-85-5; 2-thiophenecarboxaldehyde, 98-03-3; trimethoxybenzaldehyde, 86-81-7; 4-(dimethylamino)-2-methylbenzaldehyde, 1199-59-3; 2-furancarboxaldehyde, 98-01-1; benzophenone, 119-61-9.

Analogues of Growth Hormone-Releasing Factor (1–29) Amide Containing the Reduced Peptide Bond Isostere in the N-Terminal Region[1]

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Previous peptide structure–activity investigations employing the ψ[CH_2NH] peptide bond isostere have produced antagonists when inserted into various sequences. These include bombesin, in which the incorporation of Leu[13]ψ[CH_2NH]Leu[14] produced a potent antagonist, and tetragastrin, with which Boc-Trp-Leuψ[CH_2NH]Asp-Phe-NH_2 is an antagonist. In this study, we chose to investigate the effect of this isostere on growth hormone-releasing factor (1–29) amide. Analogues were prepared by solid-phase synthesis and the isosteres incorporated by racemization-free reductive alkylation with a preformed protected amino acid aldehyde in the presence of $NaBH_3CN$. The aldehydes were prepared by the reduction of the protected *N*,*O*-dimethyl hydroxamates with $LiAlH_4$ at 0 °C. The purified analogues were assayed in a 4-day primary culture of male rat anterior pituitary cells for growth hormone (GH) release. Potential antagonists were retested in the presence of GRF(1–29)NH_2. The following results were obtained: At position 5–6, a very weak agonist was produced with ≪0.01% activity. Incorporation of the isostere in positions 1–2, 2–3, and 6–7 gave weak agonists with ~0.1% activity. Agonists with 0.39% and 1.6% activity were produced by incorporation at 10–11 and 3–4, respectively. The analogue [Ser[9]ψ[CH_2NH]Tyr[10]]GRF(1–29)NH_2 was found to be an antagonist in the 10 μM range vs 1 nM GRF and had no agonist activity at doses as high as 0.1 mM.

Growth hormone-releasing factor (GRF), a 44-residue peptide, was isolated from a pancreatic tumor occurring in an acromegalic patient.[2,3] The structural characterization of GRF has resulted in numerous basic and clinical studies into this peptide's role in the control of GH secretion and its ultimate effects on growth itself. Structure-activity studies have shown that the full sequence is not required for activity and that the shortened sequence GRF(1–29)NH_2 is fully potent,[3] greatly simplifying the synthesis of analogues for further structure–activity studies. The data accumulated thus far indicate that GRF may be of value in certain clinical disorders, including childhood GH deficiency, as well as in agricultural applications pertaining to milk and meat production. In common with a number of other peptide hormones, the plasma half-life of GRF is of the order of minutes.[4] This presents a major problem in the development of potent long-acting therapeutic or agricultural agents. The most common approach to increasing the potency and duration of action of analogues has been the incorporation of unusual or D-amino acid residues in various regions of the hormone.[5-9] A less common strategy in the elaboration of structure–activity relationships is the modification of the peptide backbone by the incorporation of various peptide bond isosteres including ψ[CH_2NH], ψ[CH_2S], and ψ[CH_2CH_2].[10] Previously, the incorporation of these isosteres required the custom synthesis of modified dipeptide units with the concomitant risk of racemization of the non-urethane-protected carboxyl terminus during activation. We have developed a racemization-free, solid-phase method for the generation of the ψ[CH_2NH] isostere in situ,[11,12] which greatly facilitates the investigation of the role of the backbone in peptide activity. The method involves the reductive alkylation of the deprotected, resin-bound peptide with a protected amino acid aldehyde in the presence of $NaBH_3CN$. Structure–activity investigations with the ψ[CH_2NH] peptide bond isostere have produced antagonists when inserted into various peptides. These include bombesin, in which the incorporation of Leu[13]ψ[CH_2NH]Leu[14] produced an antagonist,[13] and tetragastrin, in which Boc-Trp-Leuψ[CH_2NH]Asp-

(1) Abbreviations used in this paper for amino acids, protecting groups, and peptides follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature and Symbols as described in *Eur. J. Biochem.* **1972**, *27*, 201 and *J. Biol. Chem.* **1975**, *250*, 3215. ψ[CH_2NH], peptide bond replaced by a CH_2NH bond.

(2) Guillemin, R.; Brazeau, P.; Bohlem, P.; Esch, F.; Ling, N.; Wehrenberg, W. *Science* **1982**, *218*, 585.
(3) Rivier, J.; Spiess, J.; Thorner, M.; Vale, W. *Nature* **1982**, *300*, 276.
(4) Froman, L. A.; Downs, T. R.; Williams, T. C.; Heimer, E. P.; Pan, Y-C. E.; Felix, A. M. *J. Clin. Invest.* **1986**, *78*, 906.
(5) Lance, V. A.; Murphy, W. A.; Sueiras-Diaz, J. A.; Coy, D. H. *Biochem. Biophys. Res. Commun.* **1984**, *119*, 265.
(6) Coy, D. H.; Murphy, W. A.; Lance, V. A.; Heiman, M. L. *Peptides* **1986**, *8* (suppl. 1), 49.
(7) Coy, D. H.; Murphy, W. A.; Lance, V. A.; Heiman, M. L. *J. Med. Chem.* **1987**, *30*, 219.
(8) Tou, J. S.; Kaempfe, L. A.; Vineyard, B. D.; Buonomo, F. B.; Della-Fera, M. A.; Baile, C. A. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 763.
(9) Rivier, J.; Rivier, C.; Galyean, R.; Yamamoto, G.; Vale, W. In *Vasoactive Intestinal Peptide and Related Peptides*; Sain, S. I., Mutt, V. Eds.; New York Academy of Science: New York, 1988; p 44.
(10) Tourwé, D. *Janssen Chim. Acta* **1985**, *3*, 3.
(11) Sasaki, Y.; Coy, D. H. *Peptides* **1987**, *8*, 119.
(12) Sasaki, Y.; Murphy, W. A.; Heiman, M. L.; Lance, V. A.; Coy, D. H. *J. Med. Chem.* **1987**, *30*, 1162.
(13) Coy, D. H.; Heinz-Erian, P.; Jiang, N-Y.; Sasaki, Y.; Taylor, J.; Moreau, J-P.; Wolfrey, W. T.; Gardner, J.; Jensen, R. *J. Biol. Chem.* **1986**, *283*, 5056.

0022-2623/90/1833-1954$02.50/0

Table I. Peptide Chromatographic, Purity, and Mass Spectral Data

peptide	analogue	HPLC 0.1% TFA eluent[a] $t_{R(major)}$/min	%	$t_{R(minor)}$/min	%	HPLC pH 3 TEAP eluent[b] $t_{R(major)}$/min	%	$t_{R(minor)}$/min	%	FAB-MS $(M - H)^+$
	GRF(1-29)NH$_2$	16.9	99.4	13.2	0.6	19.6	99.1	17.0	0.5	
I	Tyr$^1\psi$[CH$_2$NH]Ala^2	16.2	99.4	19.4	0.6	18.9	99.3	20.0	0.4	3345
II	Ala$^2\psi$[CH$_2$NH]Asp^3	14.9	94.1	12.3	3.1	18.0	95.4	15.3	3.4	3345
III	Asp$^3\psi$[CH$_2$NH]Ala^4	14.4	95.5	16.3	3.6	17.4	99.0	19.6	0.6	3345
IV	Ala$^4\psi$[CH$_2$NH]Ile^5	13.6	97.6	10.9	2.3	16.2	95.6	13.9	2.5	3345
V	Ile$^5\psi$[CH$_2$NH]Phe^6	14.3	97.2	11.4	2.8	17.1	94.8	17.8	4.5	3345
VI	Phe$^6\psi$[CH$_2$NH]Thr^7	14.7	95.3	11.9	4.2	17.7	96.8	15.0	2.6	3345
VII	Thr$^7\psi$[CH$_2$NH]Asn^8	14.7	96.7	17.1	3.1	17.5	97.8	20.2	2.0	3345
VIII	Ser$^8\psi$[CH$_2$NH]Tyr^{10}	15.8	92.6	13.1	7.3	18.5	93.9	15.1	6.0	3343
IX	Tyr$^{10}\psi$[CH$_2$NH]Arg^{11}	13.5	97.7	10.5	2.2	15.4	96.6	15.6	2.1	3344

[a] Elution system: A, 0.1% TFA; B, 0.1% TFA in 80% MeCN, 30% to 60% B at 1% min^{-1} and 1.5 mL min^{-1}. [b] Elution system: C, 5% MeCN in TEAP (0.1 M, pH = 3); D, 20% C in MeCN, 20% to 50% D at 1% min^{-1} and 1.5 mL min^{-1}.

Phe-NH$_2$ is an antagonist.[14] In this study, we investigated the effects of consecutive peptide bond replacement by the isostere in the biologically important N-terminal region of GRF(1-29)NH$_2$.

Results and Discussion

Analogues were prepared by solid-phase synthesis on methylbenzhydrylamine resin. The following protocol was used on an Advanced ChemTech ACT 200 synthesizer: deblocking, 33% TFA (1 min, 25 min); DCM wash; PrOH wash; neutralization, 10% DIEA (two washes); DMF wash; coupling of preformed HOBt esters (3 equiv formed during the deblocking reaction), 45 min in DMF, 15 min with a catalytic amount of DMAP; PrOH wash; DCM wash. The introduction of the reduced peptide bond was accomplished by the reductive alkylation of the resin-bound peptide amino terminus with a preformed protected amino acid aldehyde.[11,12] The aldehydes were prepared in two steps. Protected amino acids were reacted with *N,O*-dimethylhydroxylamine hydrochloride in DCM containing DCC and DIEA at 0 °C. The crude *N,O*-dimethyl hydroxamates were isolated by extraction followed by washing with acid and base and obtained as stable oils. When required, the aldehydes were formed by reduction of the protected *N,O*-dimethyl hydroxamates with LiAlH$_4$ at 0 °C in THF.[15] The reductions were monitored by TLC and the crude aldehydes were isolated as oils which were briefly stored at −20 °C until used. A complete description of the preparation and characterization of the aldehydes was published previously.[12] The isosteres were formed by the reductive alkylation of the preformed protected amino acid aldehyde (3 equiv) with an excess of NaBH$_3$CN in acidified DMF. The progress of the alkylation was monitored with the qualitative ninhydrin test and most reactions produced in pink or red color, which was taken as the end point. The method was shown to be free of racemization in a model study.[16] However, appreciable racemization can occur if the aldehyde is stored for a prolonged period before use. Peptide assembly was completed by using the same protocol as before. No attempt was made to block any remaining primary amino groups or to protect the secondary amino group formed during the alkylation, since previous work has shown this moiety to be unreactive during subsequent coupling reactions.[11,17] The peptides were cleaved with HF and purified by gel filtration and RP-HPLC to a final purity of >92.5% as judged by analytical RP-HPLC (see Table I).[18] All but one of the peptides were synthesized without problems and gave satisfactory amino acid analyses and the expected FAB-MS value within the error of the methods (see Table I and Table II). The analogue [Asn$^8\psi$[CH$_2$NH]Ser^9]-GRF(1-29)NH$_2$ could not be synthesized. The reaction of Boc-Asn(Xan)CHO with Ser(Bzl)~[resin] was very slow and no appreciable reaction was noted after several prolonged alkylations. This lack of reactivity may be due to the steric hindrance of the bulky side-chain protecting groups since, in a series of substance P analogues, Boc-Gln(Xan)CHO was used successfully.[19] No problems were experienced with the preparation or use of Boc-Asp-(OChx)CHO and no evidence of over reduction to homo-Ser was observed.

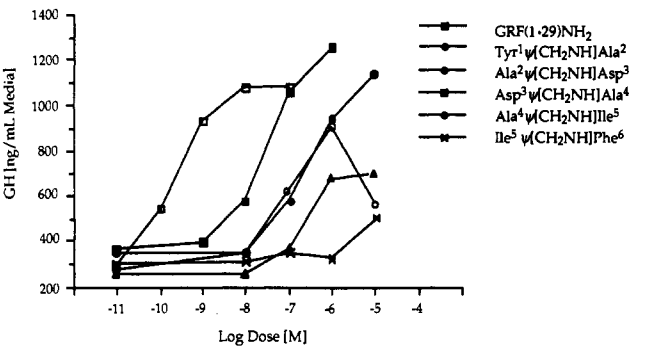

Figure 1. Effect of reduced peptide bond GRF(1-29)NH$_2$ analogues on GH secretion from dispersed rat pituitary cells.

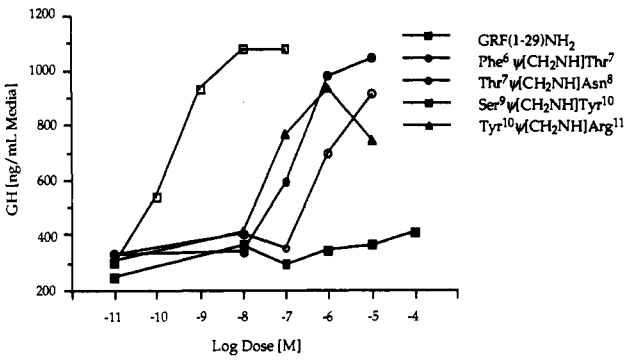

Figure 2. Effect of reduced peptide bond GRF(1-29)NH$_2$ analogues on GH secretion from dispersed rat pituitary cells.

(14) Martinez, J.; Bali, J-P.; Rodriguez, M.; Casto, B.; Magous, R.; Laur, J.; Lignon, M-F. *J. Med. Chem.* **1985**, *28*, 1874.
(15) Fehrentz, J.-A.; Castro, B. *Synthesis* **1983**, 676.
(16) Coy, D. H.; Hocart, S. J.; Sasaki, Y. *Tetrahedron* **1988**, *3*, 835.
(17) Hocart, S. J.; Nekola, M. V.; Coy, D. H. *J. Med. Chem.* **1988**, *31*, 1820.
(18) TLC data is not presented since the method has very poor resolving power with these large peptides and consistently produces single spots with the crude analogues.
(19) Qain, J. M.; Coy, D. H.; Jiang, N-Y.; Gardner, J.; Jensen, R. T. *J. Biol. Chem.* **1989**, 16667.

Table II. Amino Acid Analyses

	GRF (1–29)NH_2	I	II	III	IV	V	VI	VII	VIII	IX
Asp	3.22	3.02	1.95	1.88	2.89	2.37	3.00	1.90	2.39	3.25
Thr	1.06	1.00	0.87	0.87	1.08	0.68			0.98	0.97
Ser	2.98	2.82	2.62	2.32	3.19	2.75	2.95	2.94	1.85	2.81
Glu	2.25	2.20	2.50	1.81	2.24	2.19	2.25	2.09	2.25	2.10
Gly	1.12	1.10	1.04	1.01	1.03	1.13	0.95	0.97	1.08	1.00
Ala	3.30	2.19	2.23	2.03	2.20	1.80	3.03	3.14	3.13	3.07
Val	0.75	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Met	0.99	1.13	1.00	0.91	0.98	1.00	1.01	0.96	0.89	0.97
Ile	1.77	1.87	1.83	1.59	1.08	1.06	1.11	1.57	1.90	1.88
Leu	4.00	4.00	3.75	2.92	4.12	4.04	4.05	3.38	4.08	4.06
Tyr	1.92	1.11	1.65	1.73	2.04	1.23	2.02	2.00	0.94	0.92
Phe	0.70	0.91	0.94	0.86	0.90			0.93	0.94	1.07
Lys	2.02	1.81	1.63	2.37	2.15	2.08	1.97	2.01	2.24	1.82
Arg	3.16	2.91	2.90	2.89	3.11	3.14	3.10	3.07	3.40	2.14

Table III. In Vitro Biological Potencies of Reduced Peptide Bond GRF(1–29)NH_2 Analogues

peptide	analogue	% potency	95% confidence interval	n^a
	GRF(1–29)NH_2	100	–	28
I	$Tyr^1\psi[CH_2NH]Ala^2$	0.12	0.07–0.22	7
II	$Ala^2\psi[CH_2NH]Asp^3$	0.13	0.08–0.24	7
III	$Asp^3\psi[CH_2NH]Ala^4$	1.6	0.9–3.0	4
IV	$Ala^4\psi[CH_2NH]Ile^5$	0.02	0.01–0.05	4
V	$Ile^5\psi[CH_2NH]Phe^6$	<0.01	nd	7
VI	$Phe^6\psi[CH_2NH]Thr^7$	0.13	0.07–0.25	7
VII	$Thr^7\psi[CH_2NH]Asn^8$	0.02	0.01–0.03	4
VIII	$Ser^9\psi[CH_2NH]Tyr^{10}$	–	–	5
IX	$Tyr^{10}\psi[CH_2NH]Arg^{11}$	0.39	0.23–0.67	6

[a] n = number of separate experiments in quadruplicate from which the corresponding curves in the figures were calculated.

The purified analogues were assayed in a 4-day primary culture of male rat anterior pituitary cells for GH release as previously described using GRF(1–29)NH_2 as a control.[20] The results are presented graphically in Figures 1 and 2. The potency estimates from a four-point assay are given in Table III. Potential antagonists were retested in the presence of GRF(1–29)NH_2 (1 nM). The following results were obtained: All analogues were found to be much less active than GRF(1–29)NH_2 to a surprising degree. Incorporation of the isosteres $Tyr^1\psi[CH_2NH]Ala^2$ and $Ala^2\psi[CH_2NH]Asp^3$ gave weak agonists with ~0.1% of the activity of the control (I, $Tyr^1\psi[CH_2NH]Ala^2$, 0.12% and II, $Ala^2\psi[CH_2NH]Asp^3$, 0.13%, respectively; see Table III). At position 3, the isostere $Asp^3\psi[CH_2NH]Ala^4$ (peptide III) produced the most potent agonist of the series, which retained 1.6% of the activity of the control. When $Ala^4\psi[CH_2NH]Ile^5$ was incorporated at position 4 (peptide IV), the activity dropped to 0.02% of that of GRF(1–29)NH_2. This drop continued at position 5, where the isostere $Ile^5\psi[CH_2NH]Phe^6$ (peptide V) produced the least active agonist with a potency of <0.01% of that of the control. $Phe^6\psi[CH_2NH]Thr^7$ (peptide VI) produced an increase in the potency of 0.13% of that of GRF(1–29)NH_2, but the isostere $Thr^7\psi[CH_2NH]Asn^8$ produced a weak agonist (VII, 0.02% potency). With the isostere $Ser^9\psi[CH_2NH]Tyr^{10}$ in the peptide (VII), all trace of agonist activity was lost at doses as high as 0.1 mM. Another agonist was produced with $Tyr^{10}\psi[CH_2NH]Arg^{11}$ in the peptide although it too had low potency (IX, 0.39%). Complete dose–response curves were not be obtained for peptides IV, V, and VIII because of the high dosages required.

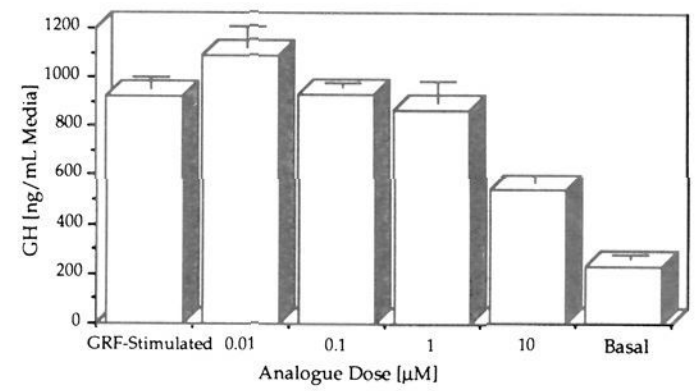

Figure 3. Antagonism of GRF-stimulated GH secretion by $[Ser^9\psi[CH_2NH]Tyr^{10}]GFR(1–29)NH_2$.

Since the analogue $[Ser^9\psi[CH_2NH]Tyr^{10}]GRF(1–29)NH_2$ (peptide VII) was found to be inactive in the potency assay, it was tested for antagonist activity in the presence of a stimulating dose of GRF(1–29)NH_2 (1 nM). The results are shown graphically in Figure 3. This analogue, $[Ser^9\psi[CH_2NH]Tyr^{10}]GRF(1–29)NH_2$, was found to be an antagonist in the 10 μM range vs 1 nM GRF and had no agonist activity at doses as high as 0.1 mM.

The loss of potency at each position was far greater and more general than that seen with smaller peptides such as somatostatin and bombesin which contain β-bends in the region of the molecule important for receptor recognition. Chou-Fasman and NMR NOE analysis[21] shows GRF to be predominantly α-helical in character in the biologically important N-terminal portion of the molecule. The replacement of CO by CH_2 has pronounced effects on α-helical formation due to a loss of intramolecular H-bonding sites and increased rotational freedom about the isostere C–N bond. Loss of intermolecular H-bonding sites might also induce changes in receptor binding capabilities of both β-bend and α-helical peptides. However, given the dramatic loss in activity of these GRF analogues as compared to that of the previously mentioned peptides, effects on peptide conformation are probably more important in the larger helical GRF molecule.

The incorporation of the reduced peptide bond isostere in the N-terminal region of GRF(1–29)NH_2 produced very weak agonists and one antagonist with an IC_{50} of approximately 10 μM. Earlier conventional structure–activity studies with the same peptide had elucidated a more potent antagonist, namely [*N*-Ac-Tyr^1,D-Arg^2]GRF(1–29)NH_2.[22] This analogue had an IC_{50} of approximately 1 μM in an assay for adenylate cyclase activity in rat anterior

(20) Murphy, W. A.; Coy, D. H. *Pept. Res.* **1988**, *1*, 36.

(21) Clore, G. M.; Martin, S. R.; Gronenborn, A. M. *J. Mol. Biol.* **1986**, *191*, 553.

(22) Robberecht, P.; Coy, D. H.; Waelbroeck, M.; Heiman, M. L.; De Neef, P.; Camus, J-C.; Christophe, J. *Endocrinology* **1985**, *117*, 1759.

pituitary homogenates. Further work is necessary to combine both antagonist modifications in an attempt to increase the potency of the analogue. The plasma enzyme responsible for degradation of GRF is a dipeptidyl-peptidase which cleaves the 2–3 amino acid bond, rather than sequential aminopeptidase cleavages.[23] Thus the incorporation of the enzyme-resistant reduced peptide bond at position 2–3 may confer enhanced enzymatic stability when combined with the superagonist or antagonist modifications although the associated profound loss of activity may be maintained in these analogues.

Experimental Section

Materials. 4-Methylbenzhydrylamine hydrochloride resin[24] (0.41 mequiv g^{-1}) was obtained from Advanced ChemTech Inc., Louisville, KY. *tert*-Butoxycarbonyl (Boc) protected amino acids were purchased from Bachem Inc., Torrance, CA or Advanced ChemTech Inc. The reactive side chains of the amino acids were masked as follows: Arg, N^g-tosyl; Asn, xanthenyl; Asp and Glu, *O*-cyclohexyl ester; Lys, N^ϵ-(2-chlorobenzyloxycarbonyl); Ser and Thr, *O*-benzyl; Tyr, *O*-(2,6-dichlorobenzyl). All reagents and solvents were ACS grade or better and used without further purification.

Amino Acid Aldehydes. The protected amino acid aldehydes were prepared in two steps by using a modification of the method of Fehrentz and Castro:[15] the protected amino acids were converted to the corresponding *N,O*-dimethyl hydroxamates by reaction with an excess of *N,O*-dimethylhydroxylamine hydrochloride (1.1 equiv) and dicyclohexylcarbodiimide (DCC, 1.1 equiv) in dichloromethane containing an excess of diisopropylethylamine (DIEA, 4 equiv) at 0 °C. The reaction was allowed to warm up to ambient temperature over 16 h with stirring. The crude, *N,O*-dimethyl hydroxamates were isolated as oils after washing with 3 M HCl (3 × 30 mL), 3 M NaOH (3 × 30 mL), and water (3 × 30 mL), drying over $MgSO_4$, and evaporation to dryness at reduced pressure. The *N,O*-hydroxamates were then reduced with $LiAlH_4$ in tetrahydrofuran at 0 °C. The reaction was followed by TLC and worked up as above to give the crude protected amino acid aldehydes.

Peptide Synthesis. The parent GRF(12–29)peptidyl-resin was assembled on 4-methylbenzhydrylamine functionalized, 1% cross-linked polystyrene resin (0.41 mequiv g^{-1}) on a 2-mmol scale by utilizing an Advanced ChemTech ACT 200 synthesizer and the following protocol: deblocking, 33% TFA (1 min, 25 min); DCM wash cycle; PrOH wash cycle; neutralization, 10% DIEA (2 wash cycles); DMF wash cycle; coupling or preformed HOBt esters (formed during deprotection), 45 min in DMF, 15 min DMAP; PrOH wash cycle; DCM wash cycle. Coupling reactions were monitored qualitatively with the ninhydrin test.[25] The peptidyl-resin was divided into aliquots, and the various analogues were then assembled on a 0.25-mmol scale. The reduced peptide bonds were formed by the reductive alkylation of the deprotected N^α-amino group with the appropriate protected amino acid aldehyde (3.0 equiv) in the presence of $NaBH_3CN$ (10 equiv) in DMF (25 mL) containing 1% acetic acid at ambient temperature for 16 h.

Peptide Cleavage. The peptides were cleaved from the resin support with simultaneous side-chain deprotection by acidolysis using anhydrous hydrogen fluoride containing anisole (~30% v/v) and dithiothreitol (~0.6% w/v) as scavengers for 1 h at 0 °C.

Purification. The crude peptides were subjected initially to gel permeation chromatography on Sephadex G50 (2.5 × 100 cm) with 2 M acetic acid eluent. Final purification was effected by preparative RP-HPLC on C_{18} bonded silica gel (Vydac C_{18}, 10–15 μm, 1.0 × 45 cm) eluted with a linear acetonitrile gradient with a constant concentration of trifluoroacetic acid (0.1% v/v). The linear gradient was generated with a Chromat-a-Trol Model II (Eldex Laboratories Inc.) gradient maker. The separations were monitored at 280 nm, by TLC on silica gel plates (Merck F60) and by analytical RP-HPLC. The fractions containing the product were pooled, concentrated in vacuo, and subjected to filtration. Each peptide was obtained as a fluffy, white powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed by RP-HPLC in two systems. Analytical RP-HPLCs were recorded with a Vydac C_{18} support (4.6 × 250 mm, 5 μm, 300-Å pore size, Liquid Separations Group). The two linear gradient systems were used at a flow rate of 1.5 mL min^{-1}: A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 30% B to 60% B at 1% min^{-1}; and C, 5% MeCN in TEAP (0.1 M, pH = 3); D, 20% C in MeCN, 20% D to 50% D at 1% min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide was assessed by the Rainin Dynamax HPLC Method Manager.

Amino Acid Analysis. The peptides were hydrolyzed in vacuo (110 °C; 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[26] (Pierce). Amino acid analyses were performed on the hydrolysates with an LKB 4150 analyser, equipped with an Ultropac 11 column (6 × 273 mm) and a Shimadzu C-R3A recording integrator with in-house software. The buffer sequence pH 3.20 (17.5 min), pH 4.25 (32 min), pH 10.00 (borate, 16 min) and temperature sequence 50 °C (5 min), 55 °C (5 min), 58 °C (39.5 min), 65 °C (7 min), 80 °C (17 min) were used. Standard retention times were as follows: His, 65.1; Lys, 70.1; NH_3, 74.3; Arg, 77.2 min, respectively. The results are given in Table II.

Mass Spectrometry. FAB-MS was conducted by Oneida Research Services, Inc., Whitesboro, NY using a Finnigan TSQ-70 equipped with an Ion Tech FAB gun at 6 kV with a primary current of 0.2 mA while scanning from 2800 to 3500 amu. The samples were dissolved in a "magic bullet" matrix and the results are given in Table I.

Biological Assays. Pituitary Cell Dispersion. Anterior pituitaries from adult male rats weighing 200–250 g and housed under controlled conditions (lights on from 0500–1900 h) were dispersed by using an aseptic technique of a previously described trypsin/DNase method[27] derived from other methods.[28,29]

Cell Culture. The dispersed cells were diluted with sterile-filtered Dulbecco's modified Eagle medium (MEM)(Gibco Laboratories, Grand Island, NY (GIBCO)), which was supplemented with fetal calf serum (2.5%; GIBCO), horse serum (3%; GIBCO), fresh rat serum (10%; stored on ice for no longer than 1 h) from the pituitary donors, MEM nonessential amino acids (1%; GIBCO), gentamycin (10 ng/mL; Sigma), and nystatin (10000 units/mL; GIBCO). The cells were counted with a hemacytometer (approximately 2 × 10^6 cells per pituitary) and randomly plated at a density of 200000 cells per well (Co-star cluster 24; Rochester Scientific Co., Rochester, NY). The plated cells were maintained in the above Dulbecco's medium in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 96 h.

In Vitro Incubation. In preparation for a hormone challenge, the cells were washed three times with medium 199 (GIBCO) to remove old medium and floating cells. Each dose of secretagogue (diluted in siliconized test tubes) was tested in quadruplicate wells in medium 199 (total volume of 1 mL) containing BSA (1%; fraction V; Sigma Chemical Co., St. Louis, MO). Cells were pulsed in the presence of somatostatin (0.1 nM) to maintain control levels within narrow limits and to increase the ratio of maximally stimulated levels of basal secretory levels without adding additional growth factors or glucocorticoids. After 3 h at 37 °C in an air/carbon dioxide atmosphere (95%/5%), the medium was removed and stored at −20 °C until assayed for hormone content.

GH RIA. GH in plasma and media was measured by a standard double antibody radioimmunoassay using components generously supplied by the NIDDK and the National Hormone

(23) Frohman, L. A.; Downs, T. R.; Heimer, E. P.; Felix, A. M. *J. Clin. Invest.* **1989**, *83*, 1533.
(24) Matsueda, G. R.; Stewart, J. M. In *Peptides: Chemistry, Structure and Biology*; Walter, R., Meinenhofer, J., Eds.; Ann Arbor Science: Ann Arbor, MI, 1975; p 333.
(25) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(26) Simpson, R. J.; Neuberger, M. R.; Lin, T. Y. *J. Biol. Chem.* **1976**, *251*, 1936.
(27) Heiman, M. L.; Nekola, M. V.; Murphy, W. A.; Lance, V. A.; Coy, D. H. *Endocrinology* **1985**, *116*, 410.
(28) Ben-Jonathan, N.; Peleg, E.; Hoefer, M. T. *Methods Enzymol.* **1983**, *103*, 249.
(29) Hoefer, M. T.; Heiman, M. L.; Ben-Jonathan, N. *Mol. Cell. Endocrinol.* **1984**, *35*, 229.

and Pituitary Program, University of Maryland School of Medicine. Data are plotted as the mean values for a given dose of peptide obtained by pooling the means from individual experiments done in quadruplicate. The number of experiments for each analogue is given in Table III. Potencies and 95% confidence intervals were calculated by four-point assay.[30]

(30) Pugsley, L. I. *Endocrinology* **1946**, *39*, 161.

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Cyclohexane Diester Analogues of Phorbol Ester as Potential Activators of Protein Kinase C†

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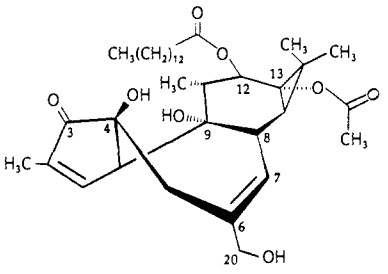
Phospholipid-dependent, Ca^{2+}-sensitive protein kinase (protein kinase C) is activated by the plant product phorbol ester at nanomolar concentrations and also in vivo at micromolar concentrations by diacylglycerols. We designed and synthesized cyclohexane diester analogues of the phorbol ester C ring as potential high-affinity activators of protein kinase C. We proposed that the necessary pharmacophore of phorbol ester could be mimicked by diesters of appropriately substituted cyclohexanediols. A series of 1,2-cyclohexanediol diesters with different substituents at position 4 was synthesized. These substituents were designed to mimic the 6,7-double bond and C-20 hydroxy of phorbol ester. Competitive binding vs [^{3}H]phorbol dibutyrate determined that these compounds have an affinity for protein kinase C of 1 mM or more, and thus they do not bind to nor are they activators of this enzyme.

Phorbol esters are tumor promoters which bind to and activate protein kinase C (PKC).[1] The activators of this enzyme that regulate it in vivo are diacylglycerols which are released upon receptor-mediated cleavage of inositol phospholipids.[2] Diacylglycerol binds to and activates PKC in concert with Ca^{2+} and membrane-associated phosphatidyl serine.[3] This activation is short-lived and well-controlled by the quick inactivation of diacylglycerol by diacylglycerol kinase[4] or diacylglycerol lipase.[5] The active phorbol esters, like TPA (1), all bind to and induce PKC



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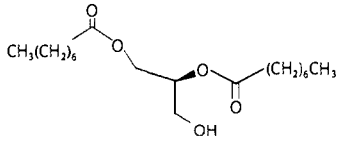

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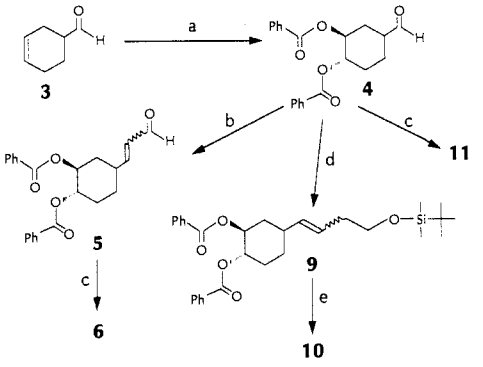
activity at nanomolar concentrations,[1f] whereas the diacylglycerols known to bind to PKC, like diC_8 (2), have affinities of 1–100 μM.[6] Phorbol esters thus can activate PKC for a prolonged period, leading to abnormally high levels of phosphorylated proteins.[7] This could be the reason for the cocarcinogenesis seen with tumor promoters. This enzyme is extremely important in signal transduction,

Scheme I. Synthesis of 1-Substituted-3,4-bis(benzoyloxy)cyclohexanes[a]



[a] (a) Silver benzoate, I_2; (b) (triphenylphosphoranylidene)acetaldehyde; (c) DBATO, PMHS; (d) butyllithium, compound 8; (e) tetrabutylammonium fluoride.

† Abbreviations used are as follows: PKC, protein kinase C; TPA, tetradecanoylphorbol acetate; diC_8, 1,2-dioctanoylglycerol; DBATO, dibutylacetyltin oxide; PMHS, poly(methoxyhydrosilane); PDBu, phorbol dibutyrate.

(1) (a) Ashendel, C. L. *Biochim. Biophys. Acta* **1985**, *822*, 219. (b) Nishizuka, Y. *Science* **1986**, *233*, 305. (c) Takai, Y.; Kaibuchi, K.; Tsuda, T.; Hoshiyima, M. *J. Cell. Biochem.* **1985**, *29*, 143. (d) Hecker, E. *Arzneim.-Forsch.* **1985**, *12*, 1890. (e) Wolf, M.; Cuatrecasas, P.; Sahyoun, N. *J. Bio. Chem.* **1985**, *260*, 15718. (f) Shoyab, M.; Todaro, G. J. *Nature* **1980**, *288*, 451. (g) Niedel, J. E.; Kahn, L. J.; Vandenbark, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 36. (h) Shoyab, M.; Todaro, G. *J. Biol. Chem.* **1982**, *257*, 434. (i) Shoyab, M.; Boaze, R. *Arch. Biochem. Biophys.* **1984**, *234*, 197. (j) Nishizuka, Y. *Cancer* **1989**, *63*, 1892.

(2) (a) Hannun, Y. A.; Loomis, C. R.; Bell, R. M. *J. Biol. Chem.* **1986**, *261*, 7184. (b) DeChaffoy de Courcelles, P.; Roevens, P.; Van Belle, H. *J. Biol. Chem.* **1985**, *260*, 15762.

(3) Kishimoto, A.; Takai, Y.; Mori, T.; Kikkawa, N.; Nishizuka, Y. *J. Biol. Chem.* **1980**, *255*, 2273.

(4) Bishop, W. R.; Ganong, B. R.; Bell, R. M. *J. Biol. Chem.* **1985**, *261*, 6993.

(5) Chabbot, H.; Cabot, M. C. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3126.

0022-2623/90/1833-1958$02.50/0