IIe: mp 95–96 °C; IR ($CCl_4$) 3610 (free OH), 3480 (bonded OH), 3050 $cm^{-1}$ (vinyl C–H); MS (20 eV, $m/z$ (relative intensity)), 298 (49.2), 280 (24.1), 265 (11.8), 146 (100); NMR ($CDCl_3$) $\delta$ 0.99 (s, 3 H), 1.36 (s, 3 H), 5.97 (d, 1 H, $J$ = 9.97 Hz), 6.25 (d, 1 H, $J$ = 10.07 Hz), 6.30 (d, 1 H, $J$ = 2.66 Hz), 7.27 (d, 1 $J$ = 2.66 Hz).

**Cafestadiene (If) and Kahweadiene (IIf).** A solution containing 0.5 g (1.8 mmol) of Ib (IIb) in 40 mL of diethylene glycol and 3.4 mL of hydrazine was refluxed for 30 min. To this reaction mixture was added 0.5 g of potassium hydroxide and refluxing was continued for 1 h. The condenser was then removed and the water–diethylene glycol mixture was evaporated until the pot temperature reached 190 °C. The condenser was replaced and refluxing was continued for an additional 2.5 h. The reaction mixture was cooled and poured into ice water. The product was collected by filtration.

If: yield 125 mg (25%); after purification by prep LC (prepPak 500/silica, hexane–ethyl acetate, 5:1, v/v) mp 90–91 °C (lit.[16,18] mp 90–93 °C); MS (20 eV, m/z (relative intensity)), 270 (100), 255 (24.9), 241 (10.1), 148 (21.2); NMR ($CDCl_3$) $\delta$ 0.82 (s, 3 H), 6.2 (d, 1 H, $J$ = 1.8 Hz), 7.2 (d, 1 H), $J$ = 1.8 Hz).

IIf: yield 100 mg (20%) after purification by prep LC, mp 70–71 °C; MS (20 eV, $m/z$ (relative intensity)), 268 (50), 253 (9.3), 239 (5.9), 146 (100); NMR ($CDCl_3$) $\delta$ 0.96 (s, 3 H), 5.95 (d, 1 H, $J$ = 10 Hz), 6.24 (d, 1 H, $J$ = 10 Hz), 6.31 (s, 1 H), 7.28 (s, 1 H).

**Hydrogenation of Ia and IIa.** A solution containing 1.06 g of Ia and IIa in 150 mL of absolute ethanol and 250 mg of palladium on activated carbon was shaken overnight in a Parr hydrogenation apparatus with 10 psi of hydrogen pressure. The catalyst was removed by filtration through a Celite pad. The ethanol was removed in vacuo. The products were separated by prep LC (silica column, hexane–ethyl acetate, 1:2, v/v).

The first fraction was crystallized from ethyl acetate to give 245 mg (23%) of IIIa: mp 186.5–189 °C; MS (20 eV, $m/z$ (relative intensity)), 318 (11.8), 300 (39.5), 289 (12.1), 96 (100); NMR ($CDCl_3$) $\delta$ 0.98 (s, 3 H), 3.48 (m, 2 H), 3.8 (m, 2 H).

The yield of IVa was 350 mg (33%): mp 154.0–157 °C (Lit.[16] mp 154.5–157 °C); MS (20 eV, $m/z$ (relative intensity)), 320 (9.8), 302 (40.5); NMR ($CDCl_3$) $\delta$ 0.98 (s, 3 H), 3.48 (m, 2 H), 3.8 (m, 2 H).

To a cooled (O °C) solution containing 500 mg (1.65 mmol) IIIa or IVa in 10 mL of pyridine was added dropwise 0.170 mL (2.0 mmol) of acetyl chloride with stirring. The mixture was then allowed to warm up to room temperature and stirring was continued overnight. The reaction mixture was poured onto ice and the aqueous solution extracted three times with 10 mL of ether. The ethereal extracts were combined and washed with 6 N HCl until all the pyridine was removed. The organic layer was washed with saturated $NaHCO_3$ solution and water. The solvent was removed in vacuo. The acetate was crystallized from EtOAc–hexane.

IIIb: yield 500 mg (90%); mp 159–160 °C; MS (20 eV, $m/z$ (relative intensity)), 360 (14.2), 302 (32.9), 287 (23.2); NMR ($CDCl_3$) $\delta$ 0.98 (s, 3 H), 2.07 (s, 3 H).

IVb: yield 500 mg (90%), mp 152–154 °C (lit.[19] mp 152–154 °C); MS (20 eV, $m/z$ (relative intensity)), 362 (1.1), 344 (4.0), 289 (100), 284 (25.6); NMR ($CDCl_3$) $\delta$ 0.98 (s, 3 H), 2.07 (s, 3 H).

**Enzyme Assay.** Female ICR/Ha mice from Harlan Sprague Dawley Co., Indianapolis, IN, were used for all experiments. Mice were randomized by weight at 6–8 weeks of age, divided into groups of four to five animals each and placed on a semipurified diet ad libitum. The diet consisted of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (U.S.P. XIV), and a complete mixture of vitamins (Teklad, Inc., Madison, WI). Four to seven days later the experimental groups were given by po administration the test compound (10 $\mu$mol in 0.2 mL of cottonseed oil with 10% ethanol) once daily for 3 consecutive days. The controls received the solvent mixture only. Twenty-four hours after the last administration the mice were killed and the tissues removed. The tissues were homogenized in phosphate buffer, pH 7.5, with a Virtis homogenizer for 45 s.

An aliquot of this homogenate was immediately used for the acid-soluble SH determination. The remaining homogenate was centrifuged at 100000$g$ for 1 h. The cytosolic portion thus obtained was used for the GST activity determination.

The activity of cytosolic GST was assayed according to the method of Habig et al.[11] using 1-chloro-2,4-dinitrobenzene as the substrate. The reaction was monitored at 340 nm in a Beckman DU-8 spectrophotometer. Assays were conducted at 30 °C in 0.1 M sodium phosphate buffer, pH 6.5, in the presence of 5 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene. Complete assay mixture without enzyme was used as the control.

**Acid-Soluble Sulfhydryl Level.** The acid-soluble SH level in tissue homogenates was assayed according to the method of Ellman.[12] Aliquots of tissue homogenates were precipitated with equal volumes of 4% sulfosalicylic acid. The supernatants were assayed for the presence of free SH groups by the addition of 9 × volume of Ellman's reagent [0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid)] in 0.1 M sodium phosphate buffer, pH 8.0. The absorbance was recorded at 412 nm.

**Acknowledgment.** We thank Thomas Krick for the mass spectral determinations and Gerald Bratt for the NMR measurements. Financial support from the National Cancer Institute (USPHS Research Grant CA 37797) is gratefully acknowledged.

**Registry No.** Ia, 469-83-0; Ib, 108664-98-8; Ic, 108665-00-5; Id, 108665-02-7; Ie, 108665-04-9; If, 108665-06-1; IIa, 6894-43-5; IIb, 108664-99-9; IIc, 108665-01-6; IId, 108665-03-8; IIe, 108665-05-0; IIf, 108665-07-2; IIIa, 108665-08-3; IIIb, 108665-10-7; IVa, 108665-09-4; IVb, 108665-11-8; GST, 50812-37-8.

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# Aldosterone Antagonists. 2. New 7α-(Acetylthio)-15,16-methylene Spirolactones

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Some 15,16-methylene derivatives of the aldosterone antagonist spironolactone (1) were synthesized with the purpose of increasing the antialdosterone potency and reducing the endocrinological effects of this standard compound. By introduction of a 1,2-double bond and a 15β,16β-methylene ring in the spironolactone molecule both goals were achieved. In animal studies mespirenone (13) exhibited a threefold-greater antialdosterone potency and less than 10% of the antiandrogenic activity of spironolactone.

In a previous paper,[1] we described the influence of substituents at the 1,2-position of the steroid framework on the aldosterone antagonistic activity of 6β,7β:15β,16β-dimethylene spirolactones. It was found that the intro-

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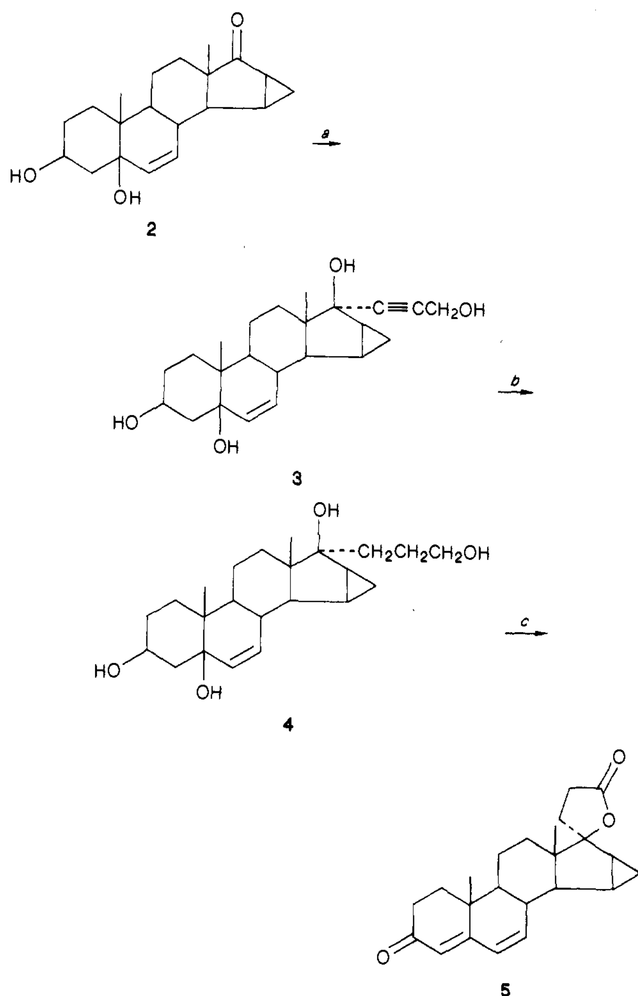
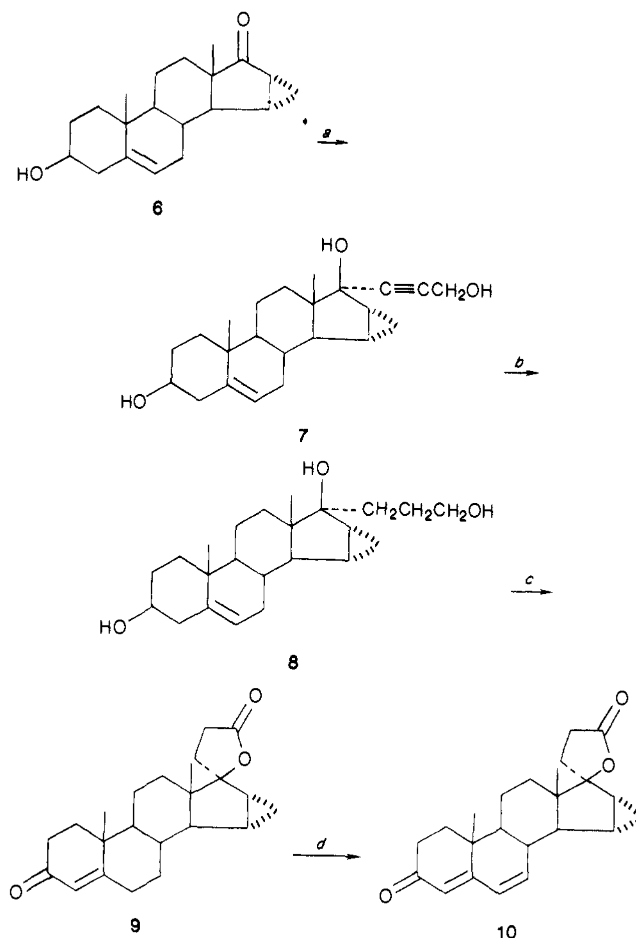
Scheme I




[a] KOEt, HC≡$CCH_2OH$, THF, room temperature. 2 h. [b] Raney nickel, $H_2$. [c] $CrO_3$, pyridine, $H_2O$, 70 °C, 18 h.

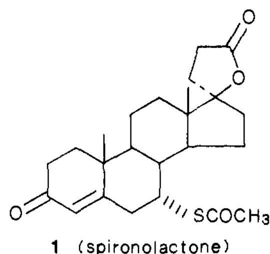
Scheme II




[a] KOEt, HC≡$CCH_2OH$, room temperature, 2.5 h. [b] Pd/$CaCO_3$/$H_2$. [c] cyclohexanone, Al(O-*i*-$C_3H_7$)$_3$, toluene, reflux 8 h. [d] (1) HC(OEt)$_3$, $H_2SO_4$, dioxane, room temperature, 1 h; (2) NBS, acetone, $H_2O$, NaOAc, 0 °C, 30 min; (3) $Li_2CO_3$, LiBr, DMF, 100 °C, 1 h.

duction of a 1,2-double bond led to a reduction of the affinity for the androgen and progesterone receptors, whereas the antimineralocorticoid activity was maintained at a high level. The 1α,2α-methylene derivatives exhibited an antimineralocorticoid activity that was comparable to that of the 1,2-dihydro or 1,2-dehydro derivatives. In contrast, the affinity for the androgen and progesterone receptors was rather high.

The structure–activity relationship known for the 6β,7β-methylene derivatives was applied to the 7α-(acetylthio) series.




1 (spironolactone)

## Chemistry

The starting material for the preparation of the new 15β,16β-methylene derivatives, compound **2**, is an intermediate of the previously reported spirorenone synthesis[2] (Scheme I). Reaction of **2** with propargyl alcohol and potassium ethylate produced the 17α-(hydroxypropynyl) derivative **3**, which could be selectively hydrogenated with Raney nickel under atmospheric pressure to **4**. The 6,7-double bond is probably protected from hydrogenation due to the cis relationship of the A/B rings of the steroid skeleton. Oxidation of the crude tetrahydroxy compound **4** with chromium(VI) oxide in aqueous pyridine at 70 °C led directly to the dienol lactone **5**.

The 15α,16α-methylene spirolactone **10** was prepared from the known 15α,16α-methylene ketone **6**[3] by a similar sequence (Scheme II). Reaction of **6** with propargyl alcohol and potassium ethylate yielded **7**, which was hydrogenated to **8** under conditions described above. Oppenauer oxidation led directly to the 3-keto lactone **9**. Introduction of the 6,7-double bond to yield **10** was accomplished in the following manner: The α,β-unsaturated ketone **9** was converted to the 3,5-dienol ether and brominated with *N*-bromosuccinimide. Dehydrobromination with lithium bromide and lithium carbonate afforded **10**.

The 7α-(acetylthio) derivatives **11–14** were synthesized from either **5** or **10** by nucleophilic addition of thioacetic


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**Table I.** Biological Activities of Spirolactones

| compd | antialdosterone act.:[a] maximal rel potency (spironolactone = 100; 95% CL) | androgen receptor competition factor[b] (dihydrotestosterone = 1.0) | progesterone receptor competition factor[b] (progesterone = 1.0) |
|---|---|---|---|
| 1 | 100 | 8.9 | 21 |
| 11 | 186 (156–245) | 41 | 14 |
| 12 | 282 (167–420) | 43 | 18 |
| 13 | 333 (210–439) | 103 | 33 |
| 14 | 115 (66–130) | 192 | 76 |
| 19 | 228 (204–257) | 15 | 7.8 |
| 20 | 281 (204–303) | 16 | 19 |

[a] Adrenalectomized, glucocorticoid rats were iv infused with aldosterone. Test compounds were administered orally 1 h prior to start of the infusion. Urine was collected from the first until the 15th hour of infusion, and urinary Na and K concentration was measured. Based on the urinary Na/K ratio and the log (Na × 100)/K ratio, the relative potency of test drug vs. standard was calculated for each hour by using regression analysis. The given data refer to the hour of maximal relative potency of test drug vs. standard. [b] The competition factor is defined as the ratio of the concentration of the test compound that causes a specific displacement of the radiolabeled standard from the receptor vs. the concentration of the unlabeled standard that causes an equivalent displacement of the radiolabeled standard.

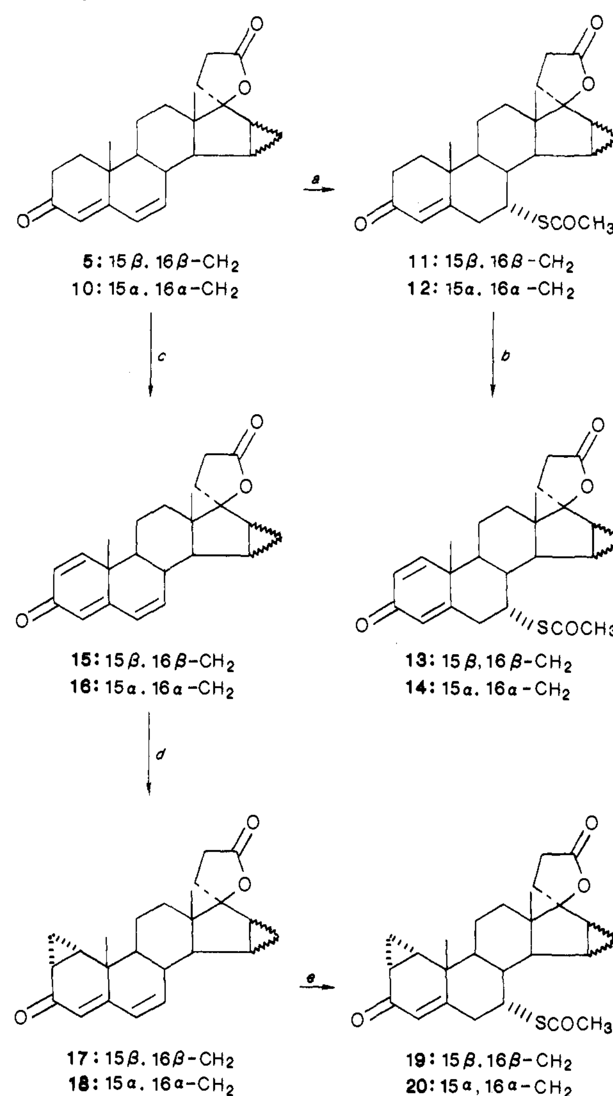
acid, followed by dehydrogenation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The $1\alpha,2\alpha$-methylene derivatives **19** and **20** were obtained from either **5** or **10** by DDQ dehydrogenation and Corey methylenation[4] followed by addition of thioacetic acid (Scheme III).

## Biological Results and Discussion

With the exception of compound **14**, all the tested compounds showed a higher antialdosterone activity than spironolactone (**1**) (Table I). These results are consistent with our previous findings in the $6\beta,7\beta$-methylene spironolactone series, in which also the $15\beta,16\beta$-methylene spirolactones exhibited higher antialdosterone activities than the corresponding 15,16-unsubstituted compounds.[1] More detailed results on the pharmacology of these compounds have been published elsewhere.[15]

The various structural alterations in the 1,2-position of the steroid molecule influence both the aldosterone antagonistic potency and the endocrinological profile. The introduction of the 1,2-double bond (**13**, **14**) led to a marked reduction of the affinity for the androgen receptor and, to a lesser degree, also for the progesterone receptor in vitro when compared to the methylene spirolactones **11** and **12**. This effect seems to be somewhat more pronounced in the $15\alpha,16\alpha$-methylene series than in the $15\beta,16\beta$-methylene series.

Scheme III




[a] $CH_3COSH$, $H_2O$, MeOH, 50 °C, 18 h. [b] DDQ, benzene, reflux, 24 h. [c] DDQ, dioxane, reflux, 3 h. [d] $(CH_3)_3SOI$, NaH, $Me_2SO$, 1.5 h, room

Compared to the 1,2-dihydro derivative **12**, the antimineralocorticoid potency of the 1,2-dehydro derivative **14** was rather low in the $15\alpha,16\alpha$-methylene series. This can be explained by the known reduction of the affinity for the mineralocorticoid receptor of 1,2-dehydro compared to the 1,2-dihydro spirolactones.[5] Surprisingly, in the $15\beta,16\beta$-methylene series, the dehydrogenated derivative **13** did not


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**Table II.** Antiandrogenic Activity of Aldosterone Antagonists in Orchidectomized Rats[a]

| compd | dose, mg/day | organ wt in % of Tp control (100%) | | rel potency |
|---|---|---|---|---|
| | | sem ves | prostate | |
| Subcutaneous Administration | | | | |
| spironolactone (1) | 1 | 64 | 73 | 1.0 |
| | 2 | 65 | 55* | |
| | 4 | 57* | 51* | |
| | 8 | 43* | 38* | |
| | 16 | 21* | 20* | |
| **12** | 2 | 86 | 84 | ~1.0 |
| | 4 | 62* | 55* | |
| | 8 | 50* | 39* | |
| | 16 | 39* | 25* | |
| mespirenone (**13**) | 4 | 67* | 61* | ~0.2 (sem vse), ~0.4 (prostate) |
| | 8 | 64* | 56* | |
| | 16 | 49* | 48* | |
| **19** | 4 | 44* | 44* | ~1.0 |
| | 8 | 34* | 31* | |
| | 16 | 27* | 22* | |
| **20** | 2 | 52* | 40* | ~1.0 (sem ves), ~2.4 (prostate) |
| | 4 | 34* | 27* | |
| | 8 | 27* | 26* | |
| | 16 | 37* | 21* | |
| Oral Administration | | | | |
| spironolactone (1) | 1 | 138 | 135 | 1.0 |
| | 3 | 130 | 134 | |
| | 10 | 91 | 82 | |
| | 30 | 58* | 56* | |
| | 100 | 20* | 14* | |
| mespirenone (**13**) | 1 | 133 | 131 | <0.1 |
| | 3 | 131 | 131 | |
| | 10 | 118 | 123 | |
| | 30 | 113 | 119 | |
| | 100 | 87 | 100 | |

[a] Tp = testosterone propionate, 0.1 mg/day, sc; sem ves = seminal vesicle; (*) = significant difference in comparing with Tp control (antiandrogen effect).

exhibit a decrease in the aldosterone antagonistic potency compared to the dihydro derivative **11**. The introduction of the 1$\alpha$,2$\alpha$-methylene moiety instead of the 1,2-double bond also resulted in very potent antimineralocorticoids. However, these compounds exhibited a stronger affinity for androgen and progesterone receptors than spironolactone.

Since all compounds except **14** showed a significantly higher aldosterone antagonistic activity than spironolactone, the endocrinological properties were determined. The main side effects of spironolactone, antiandrogenic[6] and progestational[7] activity, were determined by using the antiandrogen test in the castrated male rat[8] and the Clauberg assay with rabbits.[9] For the assessment of inhibitory effects on LH secretion, the ovulation inhibition test in the rat[9] was performed (Tables II and III).

The introduction of the 15$\alpha$,16$\alpha$- or 15$\beta$,16$\beta$-methylene moiety in the spironolactone molecule led not only to an increase in aldosterone antagonistic potency but also to an increase in progestational potency. The binding to the androgen receptor was slightly reduced. Contrary to this finding, the introduction of the 15$\alpha$,16$\alpha$-methylene moiety in the 17$\alpha$-ethinyl-17$\beta$-hydroxy-18-methyl-4-estren-3-one (Levonorgestrel) molecule increased the progestational activity, whereas the introduction of the 15$\beta$,16$\beta$-methylene moiety produced no effect on the progestational activity.[10] The further introduction of a 1$\alpha$,2$\alpha$-methylene moiety obviously had no influence on the antiandrogenic activity. There was, however, some influence on the progestational activity. The 1$\alpha$,2$\alpha$:15$\beta$,16$\beta$-dimethylene derivative **19** showed a remarkable decrease in the progestational activity compared to the 1,2-dihydro derivative **11**, whereas the 1$\alpha$,2$\alpha$:15$\alpha$,16$\alpha$-dimethylene derivative **20** showed approximately the same progestational activity as **12**.

**Table III.** Progestational and Ovulation Inhibition Activity

| compd | Clauberg test | | | ovulation inhibn test, po | |
|---|---|---|---|---|---|
| | dose, mg | McPhail index | | dose, mg | % inhibn |
| | | sc | po | | |
| spironolactone (1) | 10 | 1.2 | | 40 | 16 |
| | 30 | 1.6 | 1.0 | 100 | 20 |
| | 100 | 2.3 | 1.3 | | |
| | 300 | | 1.8 | | |
| 11 | 3 | 1.6 | | | |
| **12** | 10 | 2.6 | | | |
| | 30 | 3.1 | | | |
| | 100 | 3.1 | | | |
| mespirenone (**13**) | 10 | 1.2 | | 30 | 0 |
| | 30 | 1.7 | 1.0 | 100 | 20 |
| | 100 | 2.6 | 1.6 | | |
| | 300 | | 2.8 | | |
| **19** | 10 | 1.0 | | 40 | 100 |
| | 30 | 1.6 | | | |
| | 100 | 3.4 | | | |
| **20** | 10 | 2.4 | | | |
| | 30 | 3.0 | | | |
| | 100 | 3.1 | | | |

Replacing the 1$\alpha$,2$\alpha$-methylene moiety by a double bond in the 15$\beta$,16$\beta$-methylene series (**13**) resulted in progestional activity similar to that of spironolactone, whereas the antiandrogenic activity was reduced markedly. In the ovulation inhibition test, the 15$\beta$,16$\beta$-methylene lactone **13** and spironolactone showed only very weak activity at very high dosages, whereas the 1$\alpha$,2$\alpha$-methylene analogue **19**, probably due to its antigonadotrophic property, exhibited a strong effect.

The most effective A- and D-ring modification of the 7$\alpha$-(acetylthio) spirolactone series is a combination of a

1,2-double bond and a 15$\beta$,16$\beta$-methylene moiety. this compound, **13** (mespirenone), showed a decreased affinity to the androgen and progesterone receptors relative to spironolactone. In agreement with these in vitro results, the antiandrogenic activity was determined to be 20–40% after subcutaneous, and less than 10% after oral, administration compared to that of spironolactone in vivo. The progestational activity differed only slightly from that of spironolactone after subcutaneous application. After oral administration, **13** exhibited a somewhat higher progestational activity than spironolactone. The progestagenic activity of this type of steroid must be considered to be the combined activities of the administered drug and its metabolites. This has been demonstrated for spironolactone which is extensively metabolized.[11] Some metabolites of spironolactone exhibit a progestational potency comparable to the parent drug.[14] The different relative progestational potencies of compound **13** and spironolactone after subcutaneous and oral administration may therefore be explained by differences in the rate of metabolism dependent upon the manner of application. In addition, it must be emphasized that the ratio of antimineralocorticoid to progestational activity of mespirenone is significantly higher than that of spironolactone.

In animal studies, compound **13** has been shown to exhibit higher antialdosterone activity and remarkably lower antiandrogenic activity than spironolactone. Increase in antimineralocorticoid activity as well as absence of progestational activity at doses antagonizing the renal actions of aldosterone has also been demonstrated for mespirenone in human volunteers (phase I studies).[16] Clinical phase II studies will start in the very near future.

## Methods

**Determination of Antialdosterone Activity in Rats.** The methods used for evaluation of the antialdosterone activity in rats were described previously.[1,12,15] Adrenalectomized Wistar rats with a body weight of 140–160 g were substituted with 1 mg of fluocortolone caproate/kg on the day of surgery and 10 mg of fluocortolone/kg sc 1 day before the diuresis experiment. These glucocorticoid-substituted rats were infused intravenously with a saline–glucose solution (0.05% NaCl, 5% glucose) containing aldosterone (50 $\mu$g/L) at a rate of 3 mL/h for 15 h. The aldosterone antagonist was administered 1 h before the start of the aldosterone infusion. Urine excretion was measured in fractions of 1 h. Sodium and potassium concentrations in urine were determined by flame photometry. The antialdosterone activity was assessed by the ability of the compounds to reverse the aldosterone effect on the urinary Na/K ratio. The various antialdosterone derivatives and spironolactone were administered at three oral doses of 6.7, 13.4, and 26.8 mg/kg. The dose-response relationship was tested for each fraction by regression analysis after logarithmic transformation of the doses. The potency of the standard substance, spironolactone, was allocated the value of 100.

**Binding to Progesterone and Androgen Receptor.**[13] For the determination of the affinity of the test substance to the progesterone and androgen receptor isolated from the rabbit uterus and the rat prostate gland, respectively, tritiated progesterone and dihydrotestosterone were used as ligands, and the competition factors are defined as the multiple of the concentration to obtain displacement equivalent to the standard. A high competition factor indicates low binding strength, and a low competition factor indicates high affinity.

**Determination of Antiandrogenic Activity in Rats.**[8] Castrated male rats (SPF, Wistar strain) weighing about 100 g were treated once a day with varying doses of test compounds (1, 2, 4, 8, 16 mg/rat per day, sc, or 1, 3, 10, 30, 100 mg/rat per day, po) in combination with 0.1 mg of testosterone propionate (Tp)/rat per day (sc) for 12 days. Control animals obtained only 0.1 mg of Tp/rat per day (sc). One day after the last treatment (day 13), the animals were sacrificed and the fresh weights of seminal vesicles and ventral prostates were evaluated. Data obtained were subjected to regression/covariance analysis to compare the relative potencies of test compounds.

**Determination of Progestational Activity (Clauberg Test).**[9] Castrated infantile female rabbits (albino Neuseeland) weighing 800–1000 g were administered a daily subcutaneous dose of 0.5 $\mu$g of estradiol for 6 days (priming). Thereafter, the animals were treated with test compounds (total dose 3, 10, 30, 100 mg/rabbit, sc or 30, 100, 300 mg/rabbit, po) for 5 days. On day 12, the animals were sacrificed, and the uteri were excised for histological studies. The rate of the glandular development in endometrium was evaluated by using the McPhail scale (1–4: 1 = inactive, 4 = maximal development of the glands).

**Determination of Ovulation Inhibition in Rats.**[9] Mature female rats (SPF, Wistar strain) weighing about 200 g were used in this experiment. Prior to the starting of the treatment, the estrous cycles of animals were controlled over a period of three cycles by means of vaginal smears. The daily treatment of animals showing a regular 4-day cycle with test compounds (30, 40, 100 mg/rat per day, po) was initiated on the day of metestrus (day 1) and continued for 3 or 4 days. On day 4, animals were unilaterally ovariectomized, and the fallopian tube was dissected free. The tube was then microscopically examined by counting the number of ova to ascertain whether ovulation had occurred or not. When animals had had no ovulation, they were further treated on day 4. All animals were sacrificed on day 5, and the remaining tube was similarly examined as on day 4. The ovulation-inhibiting activity of test substances was expressed as the percent of animals in which ovulations were suppressed.

## Experimental Section

All melting points were determined on a Büchi 510 melting point apparatus and are uncorrected. NMR spectra were taken in $CDCl_3$ on a Bruker MX 90 or a Varian HA-100 spectrometer using tetramethylsilane as an internal standard unless otherwise stated. Ultraviolet spectra were obbtained in methanol on a Cary 14 UV spectrophotometer. Infrared spectra were obtained in KBr tablet on Perkin-Elmer Model 621 and 580 B infrared spectrophotometer. Optical rotations are specific rotations taken in $CHCl_3$ (0.5%).

**17$\alpha$-(3-Hydroxypropynyl)-15$\beta$,16$\beta$-methylene-5$\beta$-androst-6-ene-3$\beta$,5,17$\beta$-triol (3).** A solution of 25 g (0.080 mol) of **2** in 350 mL of THF was treated with 98.15 g (1.16 mol) of potassium ethylate at 0 °C under argon. To this solution was added 37.5 mL (0.644 mol) of propargyl alcohol, and stirring was continued for 2 h at room temperature. The reaction mixture was poured into ice water and neutralized with acetic acid, and the resulting solution was saturated with sodium chloride. The aqueous phase was extracted with ethyl acetate and washed with brine. The crude product was triturated with acetone and yielded 24.3 g (82.5%) of **3**: mp 233 °C; $[\alpha]^D$ +156°; IR 3400 cm$^{-1}$; NMR (pyridine-$d_5$ + $D_2O$) 5.8 (m, C-6, C-7), 4.65 (s, C-22), 4.25 (m, C-3), 1.15 (C-18, C-19) ppm. Anal. ($C_{23}H_{32}O_4$) C, H, O.

**15$\beta$,16$\beta$-Methylene-3-oxo-17$\alpha$-pregna-4,6-diene-21,17-carbolactone (5).** A solution of 65 g (0.174 mol) of **3** in 500 mL of THF and 500 mL of methanol was hydrogenated with Raney nickel under atmospheric pressure. The catalyst was filtered and the solvent evaporated to yield 65 g of **4**. The crude product was dissolved in 650 mL of pyridine and treated with a solution of

(16) Seifert, W.; Wilson, C.; Hildebrand, A. Abstract, Symposium on Corticoids and Peptide Hormones, Mannheim, Sept. 1986.

130 g of chromic anhydride in 325 mL of pyridine and 32.5 mL of water. The reaction mixture was heated for 18 h at 70 °C and poured into 7 L of ethyl acetate. The precipitate was filtered, and the filtrate was evaporated in vacuo. Chromatography on silica gel yielded 28 g (46%) of **5**: mp 168.9 °C; $[\alpha]_D$ +35.8°; UV $\epsilon_{283}$ = 26 500; IR 1770, 1665, 1620 cm$^{-1}$; NMR (pyridine-$d_5$) 6.2 (C-6, C-7), 5.85 (C-4), 0.90 (C-18, C-19) ppm. Anal. ($C_{23}H_{28}O_3$) C, H, O.

**17α-(3-Hydroxypropynyl)-15α,16α-methyleneandrost-5-ene-3β,17β-diol (7).** A solution of 26.25 g (0.087 mol) of **6** in 525 mL of THF was treated with 78.8 g (0.936 mol) of potassium ethylate at 0 °C under argon. To this solution was added 39.4 mL (0.616 mol) of propargyl alcohol, and stirring was continued for 2.5 h at room temperature. The reaction mixture was poured into ice water and neutralized with acetic acid, and the resulting solution was saturated with sodium chloride. The aqueous phase was extracted with ethyl acetate and washed with brine. The crude product was purified by chromatography on silica gel and treated with acetone to yield 26.1 g (95%) of **7**: mp 251.1 °C; $[\alpha]_D$ -174°; IR 3350 cm$^{-1}$; NMR (pyridine-$d_5$) 5.38 (d, C-6), 3.8 (m, C-3), 2.82 (d, C-4), 1.38 (C-18), 1.05 (C-19) ppm. Anal. ($C_{23}H_{32}O_3$) C, H, O.

**15α,16α-Methylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (9).** A solution of 25 g (0.0701 mol) of **7** in 250 mL of DMF and 250 mL of THF was hydrogenated with 10% palladium on calcium carbonate under atmospheric pressure. The catalyst was filtered, and the solvent was evaporated in vacuo to yield 25 g of **8**. The crude product was dissolved in 500 mL of toluene and 50 mL of cyclohexanone and treated with 12.5 g of aluminum isopropoxide and the solution heated to reflux temperature. During 8 h of reaction time, 250 mL of toluene was removed by distillation. The organic phase was diluted with ethyl acetate and washed with aqueous sulfuric acid and water. The crude product was purified by column chromatography on silica gel and recrystallized from diisopropyl ether to yield 13.6 g (54%) of **9**: mp 174.9 °C; $[\alpha]_D$ +40.4°; UV $\epsilon_{240}$ = 16 600; IR 1775, 1675, 1620 cm$^{-1}$; NMR 5.78 (C-4), 1.25 (C-18, C-19) ppm. Anal. ($C_{23}H_{30}O_3$) C, H, O.

**15α,16α-Methylene-3-oxo-17α-pregna-4,6-diene-21,17-carbolactone (10).** A solution of 11.8 g (0.0333 mol) of **9** in 130 mL of dioxane was treated with 11.8 g of triethyl orthoformate and 0.5 mL of concentrated sulfuric acid. After 1 h at room temperature, 1 mL of pyridine was added, and then the reaction mixture was diluted with ether, washed with water, and evaporated to yield 13 g of crude material. This residue was dissolved in 260 mL of acetone, 2 mL of pyridine, and 26 mL of water and treated at 0 °C with 9.82 g of sodium acetate followed by 6.8 g of *N*-bromosuccinimide. After 30 min, the solution was poured into ice water, filtered, and dried to yield 17 g of crude material, which was dissolved in 170 mL of DMF and heated with 6.84 g of lithium carbonate and 8.05 g of lithium bromide for 1 h at 100 °C. The reaction mixture was poured into water. The resulting precipitate was filtered, dried, and purified by column chromatography. Recrystallization from diisopropyl ether yielded 9 g (75%) of **10**: mp 180.5–182.5 °C; $[\alpha]_D$ -28°; UV $\epsilon_{283}$ = 26 300; IR 1775, 1665, 1620, 1585 cm$^{-1}$; NMR 6.32 (d, C-4), 6.1 (dd, C-7), 6.32 (d, C-6), 1.25 (C-18), 1.15 (C-19) ppm. Anal. ($C_{23}H_{28}O_3$) C, H, O.

**7α-(Acetylthio)-15β,16β-methylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (11).** A solution of 2 g (0.0056 mol) of **5** in 40 mL of methanol, 8 mL of water, and 4 mL of thioacetic acid was stirred for 18 h at 50 °C. After dilution with ether, the organic phase was washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel. Recrystallization from diisopropyl ether/acetone afforded 1.7 g (70%) of **11**: mp 257 °C; $[\alpha]_D$ -40°; UV $\epsilon_{235}$ = 18 700; NMR 5.78 (C-4), 4.2 (C-7), 2.35 ($SCOCH_3$), 1.21 (C-18), 1.0 (C-19) ppm. Anal. ($C_{25}H_{32}O_4S$) C, H, O, S.

**7α-(Acetylthio)-15α,16α-methylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (12).** A solution of 1.7 g (0.0048 mol) of **10** in 34 mL of MeOH, 8 mL of water, and 6.8 mL of thioacetic acid was stirred at 50 °C for 18 h. After dilution with ether, the organic phase was washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel. Recrystallization from diisopropyl ether/acetone provided 1.4 g (67%) of **12**: mp 216.6 °C; $[\alpha]_D$ -12.5°; UV $\epsilon_{238}$ = 19 100; IR 1770, 1690, 1670, 1620 cm$^{-1}$; NMR 5.70 (C-4), 4.35 (m, C-7), 2.35 (s, $SCOCH_3$), 1.22 (C-18), 1.19 (C-19) ppm. Anal. ($C_{25}H_{32}O_4S$) C, H, O, S.

**7α-(Acetylthio)-15β,16β-methylene-3-oxo-17α-pregna-1,4-diene-21,17-carbolactone (13).** A solution of 650 mg (0.0015 mol) of **11** in 65 mL of benzene was refluxed with 650 mg of DDQ for 24 h. After dilution with ether, the solution was washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel. Recrystallization from methanol gave 290 mg (44%) of **13**: mp 281 °C; $[\alpha]_D$ -82.3°; UV $\epsilon_{238}$ = 18 100; IR 1770, 1690, 1665, 1625, 1605 cm$^{-1}$; NMR 6.97 (d, C-1), 6.45 (d, C-2), 6.32 (C-4), 4.45 (C-7), 2.35 (s, $SCOCH_3$), 1.18 (C-19), 0.98 (C-18) ppm. Anal. ($C_{25}H_{30}O_4S$) C, H, O, S.

**7α-(Acetylthio)-15α,16α-methylene-3-oxo-17α-pregna-1,4-diene-21,17-carbolactone (14).** A solution of 350 mg (0.0008 mol) of **12** in 7 mL of toluene was heated with 350 mg of DDQ for 24 h at 80 °C. After dilution with ether, the solution was washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel and yielded 235 mg (67%) of **14** as an oil: $[\alpha]_D$ -26.8°; UV $\epsilon_{241}$ = 17 880; IR 1775, 1690, 1660, 1625, 1605 cm$^{-1}$; NMR 7.0 (d, C-1), 6.25 (dd, C-2), 6.05 (br s, C-4), 4.4 (m, C-7), 2.35 (s, $SCOCH_3$), 1.22 (C-18), 1.28 (C-19) ppm. Anal. ($C_{25}H_{30}S$) C, H, O, S.

**15β,16β-Methylene-3-oxo-17α-pregna-1,4,6-triene-21,17-carbolactone (15).** A solution of 12 g (0.047 mol) of **5** in 500 mL of benzene and 50 mL of dioxane was heated with 12 g of DDQ for 20 h at 80 °C. After dilution with ethyl acetate, the solution was washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel and afforded 9.5 g (79.8%) of **15** as a foam: $[\alpha]_D$ +11°; UV $\epsilon_{299}$ = 11 830, $\epsilon_{255}$ = 9645, $\epsilon_{222}$ = 11 550; IR 1770, 1650, 1600 cm$^{-1}$; NMR 7.04 (d, C-1), 6.25 (br s, C-6, C-7), 6.2 (d, C-2), 1.08 (C-18), 1.2 (C-19) ppm. Anal. ($C_{23}H_{26}O_3$) C, H, O.

**15α,16α-Methylene-3-oxo-17α-pregna-1,4,6-triene-21,17-carbolactone (16).** A solution of 5 g (0.014 mol) of **10** in 50 mL of dioxane was refluxed with 5 g of DDQ for 3 h. The reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was dissolved in ether and washed with aqueous $NaHCO_3$ and water. The crude product was purified by column chromatography on silica gel and gave 3.5 g (70%) of **16** as an oil: UV $\epsilon_{299}$ = 11 500, $\epsilon_{254}$ = 9150, $\epsilon_{222}$ = 11 250; IR 1770, 1650, 1600 cm$^{-1}$; NMR 7.0 (d, C-1), 6.22 (m, C-6, C-7), 6.2 (dd, C-2), 5.98 (br s, C-4), 1.25 (C-18), 1.2 (C-19) ppm. Anal. ($C_{23}H_{26}O_3$) C, H, O.

**1α,2α:15β,16β-Dimethylene-3-oxo-17α-pregna-4,6-diene-21,17-carbolactone (17).** A solution of 14.36 g (0.065 mol) of trimethylsulfoxonium iodide in 120 mL of dimethyl sulfoxide was stirred with 2.63 g (0.06 mol) of sodium hydride (55% suspension in oil) for 1.5 h. This solution was treated with a solution of 9.3 g (0.0265 mol) of **15** in 40 mL of dimethyl sulfoxide and the mixture stirred for 1 h at room temperature. The reaction mixture was poured into ice water and acidified with sulfuric acid. The resulting precipitate was filtered and dried. Chromatography on silica gel provided, after recrystallization from diisopropyl ether/acetone, 4.64 g (48%) of **17**: mp 208.5 °C; $[\alpha]_D$ +194°; UV $\epsilon_{282}$ = 20 400; IR 1765, 1650, 1620, 1585 cm$^{-1}$; NMR (pyridine-*d*) 6.08 (m, C-6, C-7), 5.7 (C-4), 1.08 (C-19), 0.98 (C-18) ppm. Anal. ($C_{24}H_{28}O_3$) C, H, O.

**1α,2α:15α,16α-Dimethylene-3-oxo-17α-pregna-4,6-diene-21,17-carbolactone (18).** A solution of 7.7 g (0.0348 mol) of trimethylsulfoxonium iodide in 77 mL of dimethylsulfoxide was stirred with 1.217 g (0.046 mol) of sodium hydride (55% suspension in oil) for 1.5 h. This solution was treated with 2.47 g (0.007 mol) of **16** and stirred for 2 h at room temperature. The reaction mixture was poured into ice water and acidified with sulfuric acid. The resulting precipitate was filtered and dried. Chromatography on silica gel yielded 2.1 g (82%) of **18** as foam: $[\alpha]_D$ +153°; UV $\epsilon_{281}$ = 19 500; IR 1775, 1660, 1620, 1590 cm$^{-1}$; NMR 6.21 (m, C-6, C-7), 5.49 (s, C-4), 1.28 (s, C-18), 1.25 (C-19) ppm. Anal. ($C_{24}H_{28}O_3$) C, H, O.

**7α-(Acetylthio)-1α,2α:15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (19).** A solution of 11 g (0.03 mol) of **17** in 3.5 mL of MeOH and 4.3 mL of thioacetic acid was stirred at 50 °C for 18 h. The organic phase was washed with aqueous $NaHCO_3$ and water. After dilution with ether, the crude product was purified by column chromatography on silica gel. Recrystallization from methanol afforded 7.7 g (58%) of **19**: mp

248–250 °C; $[\alpha]_D$ +88.7°; UV $\epsilon_{234}$ = 15800; IR 1775, 1690, 1660 $cm^{-1}$; NMR 5.53 (C-4), 4.17 (m, C-1), 2.38 (s, $SCOCH_3$), 1.35 (C-19), 1.02 (C-18) ppm. Anal. ($C_{26}H_{32}O_4S$) C, H, O, S.

**7α-(Acetylthio)-1α,2α:15α,16α-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone (20).** A solution of 1 g (0.0027 mol) of **18** in 20 mL of MeOH, 4 mL of water, and 1.5 mL of thioacetic acid was stirred at room temperature for 16 h. After dilution with ether, the organic phase was washed for aqueous $NaHCO_3$ and water. The crude material was purified by column chromatography on silica gel. Recrystallization from diisopropyl ether provided 460 mg (38%) of **20**: mp 246.2 °C; $[\alpha]_D$ +120°; UV $\epsilon_{235}$ = 16500; IR 1775, 1690, 1660, 1620 $cm^{-1}$; NMR 5.52 (C-4), 4.2 (m, C-7), 2.35 (s, $SCOCH_3$), 1.25 (C-19), 1.18 (C-18) ppm. Anal. ($C_{26}H_{32}O_4S$) C, H, O, S.

# Synthesis of Two Glucagon Antagonists: Receptor Binding, Adenylate Cyclase, and Effects on Blood Plasma Glucose Levels

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In diabetes mellitus, hyperglycemia is often associated with elevated levels of glucagon in the blood. This suggests that glucagon (1) is a contributing factor in the metabolic abnormalities of diabetes mellitus. A glucagon-receptor antagonist would provide direct evidence for glucagon's role in diabetes mellitus. On the basis of careful consideration of conformational, amphiphilic, and structural factors, we have synthesized two new glucagon analogues with antagonist biological activities by using solid-phase methodology. These two new analogues, [Asp[3],D-Phe[4],Ser[5],Lys[17,18],Glu[21]]glucagon (2) and [D-Phe[4],Tyr[5],3,5-$I_2$-Tyr[10],Arg[12],Lys[17,18],Glu[21]]glucagon (3), had $IC_{50}$ values 5.4% and 50% those of glucagon, respectively, and showed no measurable adenylate cyclase activity. When tested in normal rats, 2 lowered plasma glucose levels and suppressed glucagon-mediated hyperglycemia 105 ± 8%, back to basal levels. Analogue 3, which lowered the basal adenylate cyclase activity in rat liver plasma membranes, increased plasma glucose levels at very high concentration in vivo and inhibited glucagon-mediated hyperglycemia in normal rats by 50%. However, neither of the new glucagon antagonists lowered the plasma glucose levels of diabetic animals. The data would suggest these new glucagon-receptor antagonists may have two actions: (a) in normal rats they can act as standard glucagon-receptor inhibitors of glucagon-mediated glycogenolysis; (b) in diabetic rats, however, because of the low levels of glycogen in the liver, the antagonists apparently have little or no antagonist effect or enhancement on glucagon-mediated glucose production.

Glucagon,[1] a 29 amino acid peptide hormone, is produced in the α-cells of the pancreas. Its main target is the liver cell, where it increases glycogenolysis and gluconeogenesis.[2] There is evidence for the existence of high- and low-affinity receptors for glucagon in rat and canine liver,[3,4] but only one receptor protein could be isolated by photoaffinity labeling.[5] The glucagon–receptor complex activates adenylate cyclase activity,[6] and the cAMP thus produced stimulates an enzyme cascade that leads to activation of phosphorylase and the inhibition of glycogen synthesis.[7] Additionally, glucagon stimulates amino acid and lipid catabolism which results in gluconeogenesis and ketosis[2] and lowers the intracellular $Ca^{2+}$ concentration by inhibiting the $Ca^{2+}$ pump in rat liver plasma membranes.[8] In addition to the classical cAMP-mediated stimulation of glycogenolysis by glucagon, there is evidence for a cAMP-independent pathway.[9]

In diabetes mellitus, hyperglycemia and ketosis are generally associated with elevated levels of glucagon in the blood. This suggests that excessive glucagon could be a contributing factor in the metabolic abnormalities of diabetes mellitus.[10] According to the bihormonal hypothesis,[11] insulin deficiency causes impairment of glucose utilization, but overproduction of glucose and ketones by the liver is primarily mediated by glucagon. Therefore, several investigators have tried to develop glucagon-receptor antagonists that would provide direct evidence for glucagon's role in diabetes mellitus.[12–14] The understanding of structure–activity relationships that are necessary for the development of an antagonist have been primarily based on studies utilizing receptor-binding and adenylate cyclase activity with rat liver plasma membranes. In this approach, an ideal antagonist should bind to the glucagon receptors in plasma membranes with high affinity, but without having any intrinsic adenylate cyclase activity.

Utilizing this approach, we recently have reported design principles and structural considerations that have led to the synthesis of a highly potent agonist,[15] to a better un-

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations include the following: cAMP, 3′,5′-cyclic adenosine monophosphate; THG, [1-$N^{\alpha}$-trinitrophenylhistidine,12-homoarginine]glucagon; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography.

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