found in the 1% CH_3COOH extract, which was neutralized, oxidized, and lyophilized. A 338.9-mg portion (41.5%) of the crude extract was purified by gel filtration on Sephadex G-25, and fractions eluting between 342 and 396 mL pooled and lyophilized to yield 312.4 mg (92.2%) of homogeneous dimer.

Acetylcysteinylprolylprolylthreonylprolylseryl[^{3}H]prolylserinamide (HRP-64). HRP-64 was prepared by reducing HRP-21 dissolved in 50 mM Tris buffer (pH 8.5) with a fivefold excess of DTT under N_2. After 1 h at room temperature, the reaction mixture was acidified with concentrated HCl (pH 2) and extracted with AcOEt, and the aqueous phase was subjected to HPLC as described for HRP-61.

Acknowledgment. This research was supported by NIDR Grant DE-07257. Analytical techniques performed by the following people are gratefully acknowledged: amino acid analyses, Mr. G. Crombie, Department of Biochemistry, Boston University School of Medicine, Boston, MA; amino acid sequence analysis, Dr. R. F. Troxler, Department of Biochemistry, Boston University School of Medicine, Boston, MA; magnetic resonance studies, Dr. M. Blumenstein, Department of Chemistry, Hunter College, New York, NY; optical rotary dispersion, Dr. B. Holmquist, Department of Biophysics, Harvard University Medical School, Boston, MA; and optical rotation, Dr. M. Walsh, Biophysics Institute, Boston University School of Medicine, Boston, MA.

Registry No. HRP-1, 114791-17-2; HRP-2, 91417-20-8; HRP-2 (tritium labeled), 114819-71-5; HRP-18, 91417-25-3; HRP-19, 91417-24-2; HRP-20, 91417-23-1; HRP-21, 91417-22-0; HRP-25, 91417-21-9; HRP-59, 114819-68-0; HRP-61, 114819-70-4; HRP-62, 114791-18-3; HRP-63, 114791-19-4; HRP-64, 114791-20-7; HRP∞75, 114819-69-1; HRP-75 (tritium labeled), 114791-21-8.

Soft Drugs. 7. Soft β-Blockers for Systemic and Ophthalmic Use

Nicholas Bodor,* Alaaeldin A. El-Koussi,[1] Masanobu Kano,[2] and Mohamed M. Khalifa[1]

University of Florida, College of Pharmacy, Center for Drug Design and Delivery, Box J-497, J. Hillis Miller Health Center, Gainesville, Florida 32610. *Received July 27, 1987*

The "inactive metabolite approach" was used to design a series of "soft" drugs derived from the acidic metabolite of metoprolol. Pharmacokinetic and pharmacodynamic properties of these novel "soft" β-adrenoceptor antagonists were determined: half-lives in human blood ranged from 5 to 754 min. The rates of in vivo disappearance of representative slow, medium, and fast hydrolyzing esters were determined in rats. In each case rapid and quantitative conversion to the corresponding free acid was observed. This suggests a facile, one-step degradation to the predicted major metabolite. The compounds were tested for their ability to decrease intraocular pressure in a rabbit model. Five of the new compounds exerted an ocular hypotensive action comparable to or greater than that of the reference compound, timolol maleate, and with a prolonged duration of action in some cases. In contrast the new compounds showed reduced and shorter duration systemic activity. The adamantylethyl ester emerges as a potentially effective antiglaucoma agent with significantly improved site-specific activity.

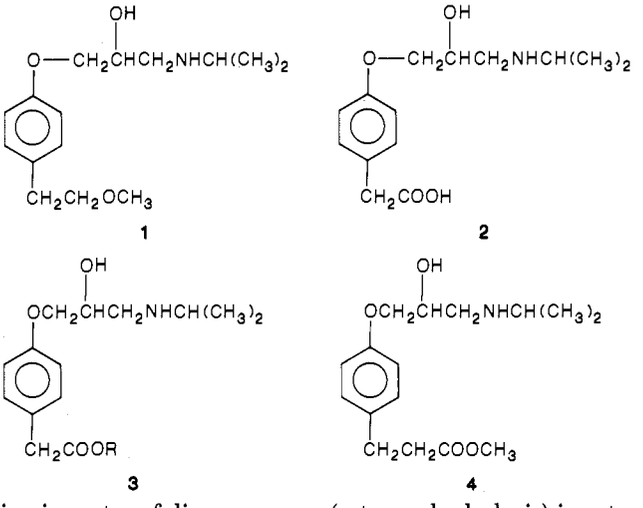
We have recently reported[3] the application of the "inactive metabolite approach"[4-6] for the design of a new class of soft β-adrenoceptor antagonists derived from metoprolol (1). According to this approach, a set of esters of the acidic metabolite 2 of metoprolol were synthesized and tested as short-acting β-blockers. The principles of the design process were previously described;[3-5] an inactive hydrophilic metabolite is converted to active derivatives which will be deactivated in a predictable and controllable way by a one-step metabolic conversion to the known inactive metabolite.

Earlier results[3] suggested that the alcohol used to derivatize 2 has a significant effect on the extent and duration of activity of the esters, whereas the rates of hydrolytic (esterase) deactivation did not seem to affect these important parameters. Thus, the more lipophilic cyclohexyl and 3,3,5,5-tetramethylcyclohexyl esters showed the highest β-blocking potency in dogs and rats, despite the fact that these compounds are rapidly hydrolyzed ($t_{1/2}$ = 1 min) in plasma.

In the present study we have further concentrated our efforts on the ester functions: a set of lipophilic alcohols were incorporated in the molecule as indicated by the general formula 3.

The potential importance of the soft drug concept was recognized by others: a short-acting β-blocker (the methyl ester of the homologue of 2, Esmolol, 4) was developed.[7] This compound 4 was also included in our studies for comparison.

All of these compounds **3a–g** were tested first for their in vitro rates of hydrolysis in human blood and for their in vivo rates of disappearance (esterase hydrolysis) in rats. Pharmacological activity studies on **3a–g** showed the effects of these compounds on resting heart rate and on

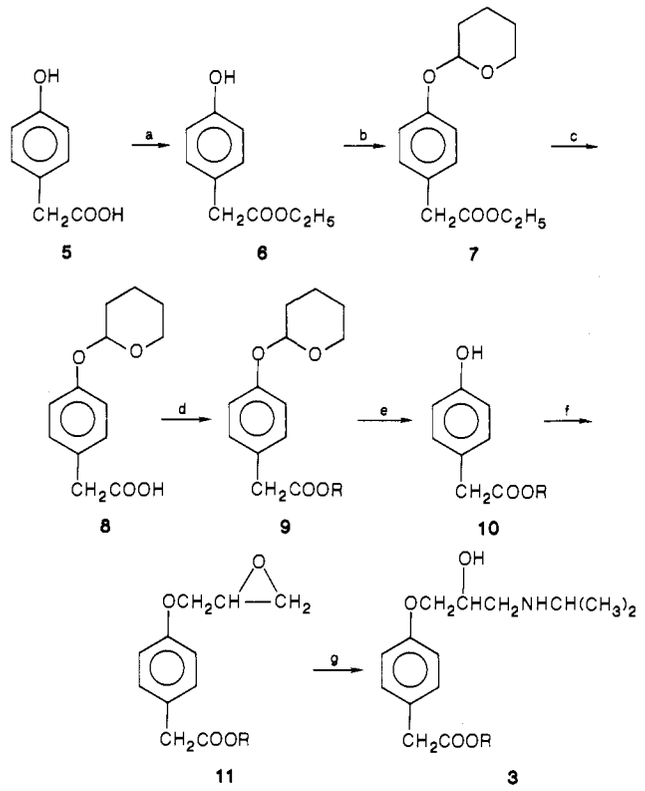


* To whom correspondence should be addressed.

(1) On leave of absence from Faculty of Medicine, University of Assiut, Assiut, Egypt.
(2) On leave of absence from Otsuka Pharmaceutical Co., Osaka, Japan.
(3) Bodor, N.; Oshiro, Y.; Loftsson, T.; Katovich, M.; Caldwell, W. *Pharm. Res.* **1984**, *3*, 120–125.
(4) Bodor, N. In *Advances in Drug Research*; Testa, B., Ed.; Academic: London, 1984; Vol. 13, pp 255–331.
(5) Bodor, N.; Kaminski, J.; Selk, S. *J. Med. Chem.* **1980**, *23*, 469–474.
(6) Bodor, N. *CHEMTECH* **1984**, *14*, 28–39.
(7) Erhardt, P. W.; Woo, C. M.; Anderson, W. G.; Gorezynski, R. J. *J. Med. Chem.* **1982**, *25*, 1408–1412.

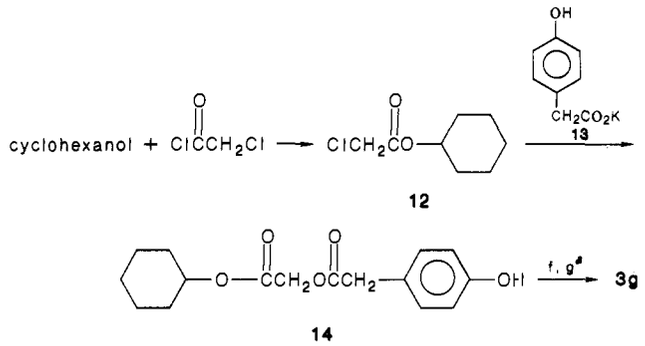
0022-2623/88/1831-1651$01.50/0

Scheme I.[a] Synthesis of Compounds **3a–f**



[a] Reagents: (a) C_2H_5OH–H_2SO_4; (b) dihydropyran–HCl; (c) CH_3OH–10% aqueous NaOH; (d) ROH, DCC, pyridine; (e) CH_3OH, 2% aqueous $(CO_2H)_2$; (f) NaH–$ClCH_2\overline{CHOCH_2}$; (g) $(CH_3)_2CHNH_2$.

Scheme II. Synthesis of **3g**



[a] See steps f, g in Scheme **I**.

isoprenaline-induced tachycardia in rats. The compounds appear to be rapidly metabolized to the inactive metabolite and as such may be suitable for systemic use in patients requiring short-duration β-blockade. Their effect on intraocular pressure (IOP) in rabbits was also demonstrated. The latter study represents a potential important use of these compounds: antiglaucoma drugs with reduced systemic activity.

Chemistry

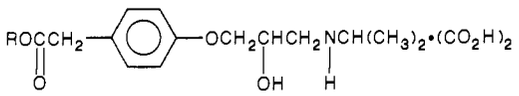
The starting material for compounds **3a–f** was 4-hydroxyphenylacetic acid (**5**). In an improvement on our previously published method,[3] it was found advantageous to first protect the phenol function during the esterification process; all intermediates prepared by using this method were homogeneous by TLC and suitable for subsequent use without further purification until the final step of the synthesis, the general scheme for which is shown in Scheme I. The easily removable 2-tetrahydropyranyl ether function was selected for protection of the phenol group. A slightly different route was used for preparation of the cyclohexylglycolyl derivative **3g** (Scheme II). In all cases, yields were in the range 75–95% and final products were fully characterized as oxalate salts.

Table I. Hydrolytic Rate Constants (k),[a] Half-Lives ($t_{1/2}$),[a] and HPLC Retention Time[b] of the β-Adrenergic Antagonists **3a–g** and **4**

no.	R	$K \times 10^3$, min^{-1} ± SE	$t_{1/2}$ min	retention time, min
3g	—CH_2COO— (H) (cyclohexylglycol)	154.00 ± 3.00	4.5	4.0
3b	CH_2CH_2- (adamantylethyl)	100.0 ± 5.00	6.9	11.0
3e	(*exo*-norbornyl)	35.0 ± 3.00	19.8	4.5
3f	(*endo*-norbornyl)	20.0 ± 0.20	34.6	4.6
3a	CH_2- (adamantylmethyl)	15.0 ± 0.60	46.2	7.4
3c	CH_3, CH_3, CH_3 (isopinocamphyl)	2.7 ± 0.06	256	7.1
3d	CH_3, H_3C, CH_3 ((1*S*)-*endo*-bornyl)	0.9 ± 0.03	770	6.2
4	(esmolol)	3.3 ± 0.08	210	3.2

[a] Pseudo-first-order constant. Values represent the mean of at least four runs ± SE of the mean. [b] Conditions described in the Experimental Section. (cyclohexylglycol) (adamantylethyl) (*exo*-norbornyl) (*endo*-norbornyl) (adamantylmethyl) (isopinocamphyl) ((1*S*)-*endo*-bornyl)

Table II. Preparation and Properties of the Soft β-Blockers **3a–h**



no.	ester	mp, °C	yield,[a] %	formula	recrystn solvent
3a	adamantylmethyl	129–131	86	$C_{27}H_{39}NO_8 \cdot {}^1/_2H_2O$	CH_2Cl_2
3b	adamantylethyl	95–96	68	$C_{28}H_{41}NO_8$	CH_2Cl_2
3c	isopincamphyl	98–100	84	$C_{26}H_{39}NO_8$	CH_2Cl_2
3d	(1*S*)-*endo*-bornyl	87–90	83	$C_{26}H_{39}NO_8$	CH_3OH
3e	*exo*-norbornyl	141–143	45	$C_{23}H_{33}NO_8$	CH_2Cl_2
3f	*endo*-norbornyl	143–146	63	$C_{23}H_{33}NO_8$	CH_2Cl_2–Et_2O
3g	cyclohexylglycolyl	131–133	54[b]	$C_{24}H_{35}NO_{10}$	CH_2Cl_2–Et_2O
3h	cyclohexyl	131–132	*c*	$C_{22}H_{33}NO_8 \cdot H_2O$	$(CH_3O)_2CO$

[a] Overall yield from **8**. [b] Overall yield from **5**. [c] Prepared as described in ref 3.

Results and Discussion

The in vitro hydrolytic stabilities of the compounds were first investigated in freshly collected human blood. As shown in Table I, the cyclohexylglycolyl **3g** and the ada-

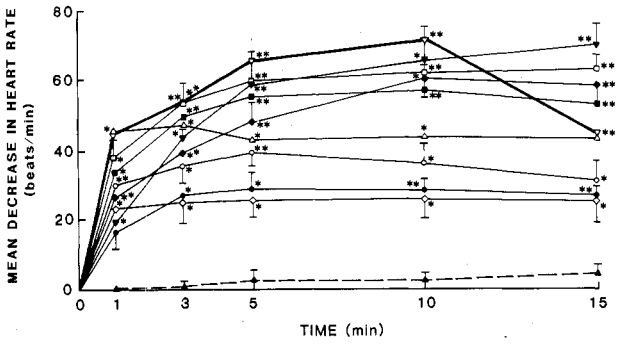


Figure 1. Effect of the iv injection of the soft β-blockers (6 mg/kg) on the resting heart rate of rats. (▽) propranolol hydrochloride, (▼) **3c**, (□) **3a**, (♦) **3b**, (■) **3d**, (△) **3e**, (○) **3f**, (●) **4**, (◇) **3g**, and (▲) saline solution. (*) Significant change ($P < 0.05$); (**) highly significant change ($P < 0.01$).

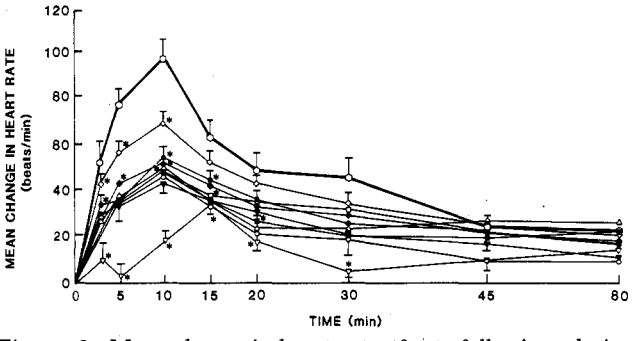


Figure 2. Mean change in heart rate of rats following administration of isoproterenol (50 μg/kg, sc). Each group was pretreated with either (▽), propranolol hydrochloride, (●) **4**, (▼) **3c**, (○) **3f**, (■) **3d**, (△) **3e**, (♦) **3b**, (◇) **3g**, (□) **3a**, or (○) saline. Each compound was administered intravenously at a dose of 6 mg/kg 15 min prior to isoproterenol. (*) Significant change ($P < 0.05$).

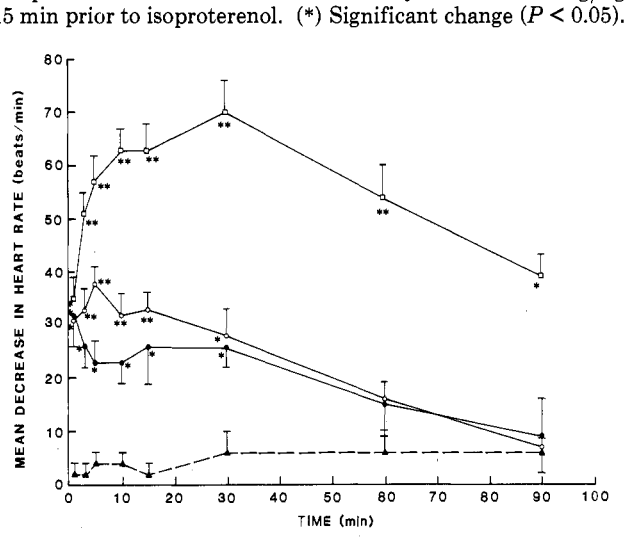


Figure 3. Duration of the effect on resting heart rate in rats following iv injection of 6 mg/kg of **3d** (□), **3g** (●), **4** (○), and saline solution (▲). (*) Significant change ($P < 0.05$); (**) highly significant change ($P < 0.01$).

mantylethyl **3b** esters are hydrolyzed the fastest and the hindered esters **3d** and **3c** the slowest, and the rates for norbornyl esters **3e** and **3f** are in between these extremes. It is not surprising that the cyclohexylglycolyl ester **3g** is hydrolyzed most rapidly, as it is actually a double ester. However, quantitative in vitro conversion of **3g** to free acid **2** was seen in this case as in all others, indicating that **3g** is hydrolyzed preferentially on the glycolate portion. Compounds similar to those described here have been shown to be very stable toward nonenzymatic hydrolysis, with half-lives of 10–13 days.[3] It is also interesting to note that the least lipophilic ester **4**, although it is a simple methyl ester, is hydrolyzed relatively slowly. Assuming that in the series **3a–g** a shorter retention time indicates lower lipophilicity, it can be seen from Table I that there is no clear correlation between lipophilicity and hydrolysis rates. This suggests that for the series **3a–g** but not for **4** the hydrolysis rates are not directly dependent on the binding properties, but rather on actual steric availability of the ester function to the enzyme. Since the initial pharmacological studies were intended to be performed in rats, it was considered important to determine the rates of hydrolysis of the test compouns in vivo, in whole animals. On the basis of the results obtained from the studies in human blood, three compounds, the slow hydrolyzing **3d** ($t_{1/2}$ = 754 min), the fast hydrolyzing **3g** ($t_{1/2}$ = 5 min), and the reference compound **4** were chosen for the in vivo study.

The study revealed that each of these compounds disappeared very quickly from the blood after iv injection. The slight differences observed (**3g** was all gone after 1 min, **3d** and **4** in 2–3 min) did not seem significant. The early appearance of the free acid **2** as the hydrolysis product was observed (but not quantitated) in all three cases. Thus, each of these β-blockers fulfills the requirements of soft drugs in vivo; that is, they undergo a facile hydrolytic deactivation to the predicted major metabolite.

Next, the β-adrenergic antagonist activity of the compounds under investigation was addressed in rats. All of the compounds tested showed negative chronotropic activity in rats for the first 15 min following iv injection (Figure 1). In most cases this activity was comparable to that of the obvious reference drug, propranolol. The (+)-isopinocamphyl **3c**, the adamantylmethyl **3a**, and the adamantylethyl **3b** esters exhibited the highest activity in the series, whereas the cyclohexylglycolyl **3g** and the reference methyl ester showed the lowest activity. That the duration of β-blocking activity of these compounds is longer than the 1–3 min the compounds survive in the rat circulation is not surprising; β-blocking activity is apparently triggered by the initial binding of these compounds to receptors and from that point on activity is not dependent on circulating drug levels. When animals were treated with compounds **3a–g** 15 min prior to sc administration of isoprenaline, significant inhibition of isoprenaline-induced tachycardia was observed at up to 35 min following injection of the test compounds (Figure 2). The cyclohexylglycolyl ester **3g** had the lowest activity in this series and showed this activity for only 10 min following administration of isoprenaline. The order of activity of the compounds studied is as follows: on resting heart rate (15 min after iv injection), propranolol > **3c** > **3a** > **3b** > **3d** > **3f** > **3e** > **4** > **3g**; on isoprenaline-induced tachycardia (20 min after sc injection of isoprenaline), propranolol > **3f** > **3e** > **3d** > **3c** > **3b** > **4** > **3g**.

These "soft" β-blockers were designed to be short acting, and so the duration of pharmacological action of three representative compounds exhibiting slow (**3d**), fast (**3g**), and intermediate (**4**) rates of hydrolytic cleavage was examined. Although each of these compounds showed a very fast rate of in vivo disappearance from rat's blood, their negative chronotropic activity persisted for up to 30 min following iv administration (Figure 3). To further examine their duration of action, the test compounds **3d**, **3g**, and **4** were administered iv to rats either 30, 60, 90, or 180 min prior to sc administration of isoprenaline. Duration of pharmacological action (effect on heartrate) was then assessed for 60 min following isoprenaline administration. Results of this set of experiments show that both the methyl and the (1*S*)-*endo*-bornyl esters (**4** and **3d**, respectively) retained an inhibitory effect on isoprenaline-induced tachycardia up to 90 min following their iv ad-

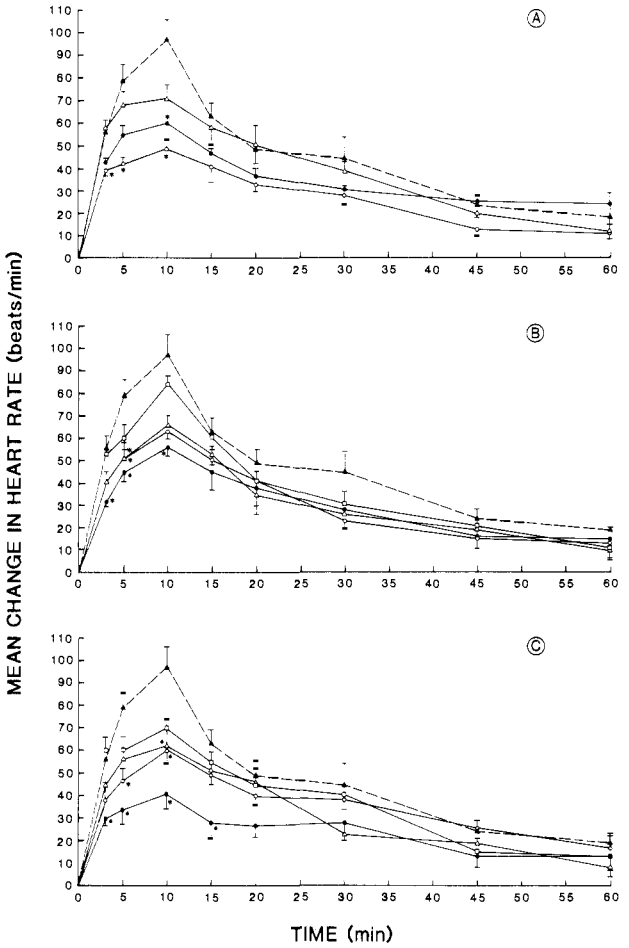


Figure 4. Duration of action of a 6 mg/kg dose of compounds **3g** (A), **3d** (B), and **4** (C) on isoprenaline-induced tachycardia in rats. Animals were treated with test compounds either 30 (○), 60 (●), 90 (△), or 180 min (□) prior to administration of isoprenaline. ▲ represents the control group, which was given saline rather than β-blocker. (*) Significant change ($P < 0.05$).

ministration, whereas the cyclohexylglycolyl ester **3g** was active for only 60 min following administration (Figure 4).

In the final set of pharmacological experiments, the effect of these new soft β-blockers on the IOP of rabbits was studied. The antiglaucoma agent timolol maleate was used as a reference drug. Five of the seven new compounds tested displayed ocular hypotensive activity which was comparable to or higher than that of timolol maleate (Figure 5). At 6 h the activity of timolol begin to diminish, whereas compounds **3f**, **3e**, **3b**, and **3c** exhibit significantly higher activity and do so for a prolonged period. Thus, 6 h following the topical administration of these compounds, a remarkable decrease in IOP was still observed. The ranking of the compounds on decrease in IOP is somewhat different at 3 and 6 h. Thus at 3 h **3f** > **3e** > **3b** > **3c** > **3a** > **4** > timolol > **3g** > **3d**, but at 6 h **3b** > **3e** > **3f** > **3d** > **3c** > timolol **4** > **3a** > **3g**. These time-dependent differences may reflect differences in intrinsic activity and metabolism or different rates of elimination from the eye. Two of the compounds, **3c** and **3d**, caused strong eye irritation, whereas **3e** and **3a** caused mild irritation. In conclusion, among the compounds tested, the adamantylethyl ester **3b** emerged as the best potential candidate for ophthalmic use, for the following reasons: (i) prolonged and significant reduction of IOP; (ii) practically no irritation to the eye, and no initial increase in IOP; (iii) very fast hydrolysis in human blood; (iv) moderate activity on heart rate and therefore a low potential for systemic side effects even if absorbed.

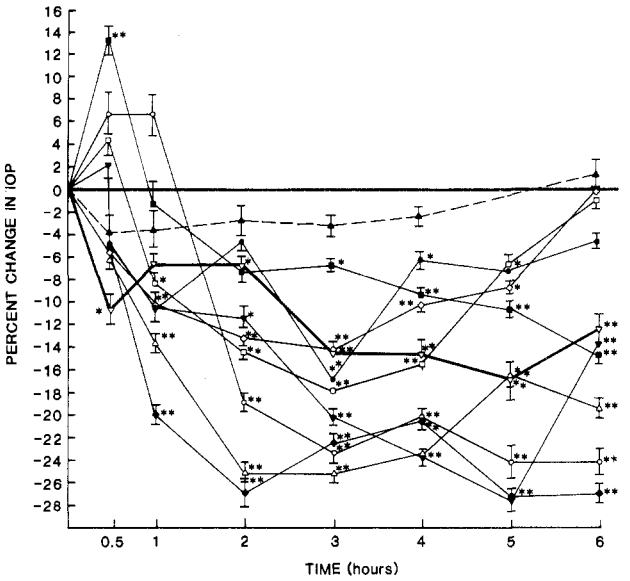


Figure 5. Effect of the topical administration of the soft β-blockers (1% solution) on the IOP of rabbits: (■) **3d**, (○) **3f**, (□) **3a**, (♦) **3b**, (△) **3e**, (◊) **3g**, (▼) **3c**, (●) **4**, (▽) timolol maleate, and (▲) saline solution. (*) Significant change ($P < 0.05$); (**) highly significant change ($P < 0.01$).

Experimental Section

All melting points are uncorrected and were obtained with an electrothermal capillary melting point aparatus. NMR spectra were recorded on a Varian EM 390 spectrometer. Elemental analyses were within ±0.4% of theoretical values. Reference compound **4** was prepared as previously described.[7] In the following, the synthesis of the *endo*-norbornyl ester **3f** is described in detail. Compounds **3a–e** were prepared in a similar manner. Unless otherwise stated, chemicals were obtained from Aldrich Chemical Co.

Ethyl 4-Hydroxyphenylacetate (6). In a round-bottomed 1-L flask were placed 4-hydroxyphenylacetic acid (**5**) (60.0 g, 0.395 mol), ethanol (750 mL), and concentrated H_2SO_4 (6 mL, 0.113 mol). The mixture was heated at reflux for 1 h and was then allowed to cool to room temperature. The mixture was further cooled in an ice bath and neutralized with dilute aqueous $NaHCO_3$ solution. The product was extracted into ethyl acetate, washed well with water, and concentrated under reduced pressure at 60 °C to yield 66.7 g (93.5%) of compound **6** as an oil. Anal. ($C_{10}H_{12}O_3$) C, H.

Ethyl 4-(2-Tetrahydropyranyloxy)phenylacetate (7). In a 1-L round-bottomed flask were placed **6** (66.7 g, 0.37 mol), ethyl acetate (270 mL), dihydropyran (10 g, 1.3 mol), and tetrahydrofuran (20 mL) saturated with HCl gas. The mixture was stirred at room temperature for 29 h. After addition of 250 mL of a 2% NaOH solution, the reaction mixture was shaken thoroughly, and the organic layer was separated, washed well with water, and dried (Na_2SO_4). Solvent was removed at reduced pressure to give 96.6 g (99%) of compound **7**. Anal. ($C_{15}H_{20}O_4$) C, H.

4-(2-Tetrahydropyranyloxy)phenylacetic Acid (8). Compound **7** (96.9 g, 0.366 mol) was placed in a 1-L round-bottomed flask containing methanol (290 mL) and 10% aqueous NaOH (290 mL). The mixture was stirred at room temperature for 18 h. After this time the mixture was cooled to −5 °C and dilute HCl was added slowly, while the temperature was maintained <5 °C. The precipitated product was collected by filtration. The filtrate was extracted with ethyl acetate. The extract and the precipitate were combined, washed well with water, dried (Na_2SO_4), and concentrated at reduced pressure to yield 75.0 g (87%) of compound **8**, mp 102–104 °C. Anal. ($C_{13}H_{16}O_4$) C, H.

***endo*-Norbornyl 4-(2-Tetrahydropyranyloxy)phenylacetate (9f).** Compound **8** (9.80 g, 41.5 mmol), *endo*-norborneol (.65 g, 41.5 mmol), pyridine (69 mL), and *p*-toluenesulfonic acid (490 mg, catalytic) were combined in a 250-mL round-bottomed flask. Dicyclohexylcarbodiimide (9.40 g, 45.7 mmol) was added, and the mixture was stirred at room temperature for 16 h, pro-

tected from moisture. The reaction mixture was then poured into water (700 mL) and was extracted with ether. The precipitated dicyclohexylurea was removed by filtration and the ether filtrate was washed twice with dilute acetic acid solution and then with water. After drying ($MgSO_4$), the extract was concentrated under reduced pressure to yield 13.0 g (95%) of compound **9f**.

***endo*-Norbornyl 4-Hydroxyphenylacetate (10f).** A mixture of **9f** (13.0 g, 39.4 mmol), methanol (104 mL), and a 2% aqueous oxalic acid solution (10 mL) was heated under reflux for 1 h, after which time it was poured into water (700 mL) and extracted with ethyl acetate. The organic extract was washed with water, dried ($MgSO_4$), and concentrated under reduced pressure at 50 °C to give the free phenol **10f** quantitatively.

***endo*-Norbornyl 4-(2,3-Epoxypropoxy)phenylacetate (11f).** A solution of **10f** (9.7 g, 39.4 mmol) in dimethylformamide (DMF) (20 mL) was placed in a 250-mL round-bottomed flask fitted with a dropping funnel under nitrogen atmosphere. The solution was cooled to −5 °C and a suspension of sodium hydride (1.7 g, 7 mmol) in DMF (29 mL) was added. After the generation of H_2 ceased, epichlorohydrin (30.8 mL, 394 mmol) was added via the dropping funnel. The mixture was stirred at room temperature for 42 h, after which time it was poured into water (500 mL) and extracted with ethyl acetate. The extract was dried ($MgSO_4$) and solvent was removed under reduced pressure to give compound **11f** quantitatively.

***endo*-Norbornyl 4-[2-Hydroxy-3-(isopropylamino)propoxy]phenylacetate, Oxalate Salt (3f).** A mixture of compound **11f** (12.0 g, 39.7 mmol), isopropylamine (23.4 g, 33.8 mL, 0.39 mol), and methanol (60 mL) was stirred at room temperature for 18 h, and then volatile material was removed under reduced pressure. The crude product (15.3 g of free base) was mixed with ether (150 mL) and a solution of oxalic acid (10.8 g, 120 mmol) in ether (100 mL). The resulting white precipitate was collected by filtration and dried at reduced pressure. The oxalate salt was further cleaned by suspending ether, stirring, and collecting by filtration. The isolated product had mp 143–146 °C.

Cyclohexyl Chloroacetate (12). Cyclohexanol (20 g, 0.2 mol), chloroform (70 mL), and pyridine (19 g, 0.24 mol) were cooled to −5 °C in a flask fitted with a dropping funnel. To this solution was added chloroacetyl chloride (27.1 g, 0.24 mol) at a rate such that the temperature of the mixture was kept below 0 °C. When addition of the acid chloride was complete, the reaction mixture was allowed to come to room temperature and was stirred for 1 h. Following this, the mixture was washed thoroughly with water and then dried ($MgSO_4$). Concentration at reduced pressure gave **12** quantitatively.

Potassium 4-Hydroxyphenylacetate (13). 4-Hydroxyphenylacetic acid (30.0 g, 0.197 mol) dissolved in 100 mL of ethanol was treated with a solution of KOH (12.8 g, 0.197 mol) in ethanol (80 mL). The mixture was stirred overnight at room temperature and the resulting precipitate was collected by filtration. After drying (60 °C, 20 mm), **12** was obtained as a white powdery solid (32 g, 85.5%), mp >300 °C. Anal. (C_8H_7OK) C, H, K.

Cyclohexylacetyl 4-Hydroxyphenylacetate (14). Cyclohexyl chloroacetate (**12**) (37 g, 0.21 mol), potassium 4-hydroxyphenylacetate (20 g, 0.105 mol), and DMSO (125 mL) were stirred together at room temperature for 24 h. The mixture was then poured into water (1.5 L) and extracted repeatedly with chloroform. After drying over Na_2SO_4, the organic phase was concentrated in vacuo to give a crude mixture containing product and excess **12**. On addition of petroleum ether the product precipitated and was collected by filtration then recrystallized from benzene to give 21.2 g (69%) of the desired product. Anal. ($C_{16}H_{20}O_5$) C, H, N. The final two steps in the synthesis of **3g** were completed as described for compounds **3a–f**.

Kinetic Studies. Analytical Method. A high-pressure liquid chromatography (HPLC) method was developed for the assay of the different compounds and their metabolites in biological fluids. The chromatographic analysis was performed on a system consisting of an LDC/Milton Roy Constametric III solvent delivery system and a Waters Lambda Max Model 481LC variable-wavelength spectrophotometer. A 30 cm × 3.9 mm (internal diameter) reverse-phase ASI C_{18} column protected with a 2.3 cm × 3.9 mm guard column packed with C_{18} Corasil packing material was used for all separations. The mobile phase consisted of 1-heptanesulfonic acid (1 g), 0.1 M acetic acid (10 mL), 0.1 M triethanolamine (100 mL), and acetonitrile (799 mL). At a flow rate of 2 mL/min the retention time of the test compounds ranged for 3.1 to 11.0 min (Table I). All solvents and reagents were of HPLC grade. Water was purified by passing through an ion-exchange bed and then distilling.

Determination of the Enzymatic Cleavage Rates in Human Blood. Freshly collected blood was obtained from Civitan Regional Blood Center Inc. (Gainesville, FL) and was used on the same day it was collected. A 100-μL volume of a freshly prepared solution of the test compound in ethanol was added to 10 mL of blood (previously equilibrated at 37 °C) and mixed thoroughly to result in an initial concentration of 10^{-3} mol/L. One-milliliter samples of blood were withdrawn from the test medium, mixed immediately with 3.0 mL of ice-cold acetonitrile, and centrifuged, and the supernatant was analyzed by HPLC.

Determination of the in Vivo Rate of Disappearance of the Compounds from Rat's Blood. Groups of at least five male Sprague–Dawley rats weighing 150–250 g were used. Animals were injected intrajugularly with the test compounds at a dose of 20 mg/kg. After 1, 2, 3, 5, 7, 10, 12, 15, 30, and 60 min a 100-μL sample of blood was withdrawn from the jugular vein and dropped immediately into a tared tube containing 1 mL of ice-cold acetonitrile. The tubes were shaken vigorously, centrifuged, and decanted, and samples were analyzed by HPLC.

Pharmacological Studies. Effect on the IOP of Rabbits. Adult male New Zealand albino rabbits weighing 2.5–3.5 kg were used. Animals were kept in individual cages with free access to food and water. IOP was measured with a Digilab Model 30R pneumatonometer. The pneumatonometer readings were calibrated at least twice a day. All measurements were obtained from unrestrained, unanesthetized rabbits. One drop of propacaine (Ophthetic-Allergen Pharmaceuticals, Inc.) diluted 1:2 with saline was instilled in each eye immediately prior to IOP measurement. Drugs (100 μL) were administered as 1% solutions in saline in both eyes of a group of at least four rabbits. Another group of at least three rabbits served as control and received only vehicle. IOP was recorded after 30, 60, and 90 min and then after 2, 3, 4, and 6 h following administration of the drug or vehicle. All IOP measurements reported were recorded by the same operator using the same tonometer. Values are given as means ± standard error of the mean. Significance of the change in IOP was determined by using the Student's t test.

Effect on Resting Heart Rate and on Isoprenaline-Induced Tachycardia in Rats. Groups of seven male Sprague–Dawley rats weighing 150–250 g were used. Each animal was anesthetized with sodium pentobarbital (50 mg/kg) and the jugular vein was cannulated with PE50 tubing. This cannula was subcutaneously threaded around the neck and exteriorized dorsally. Cannulae were filled with heparin solution (1000 units/mL) and sealed with a solid 22-guage stylet. Animals were housed in individual stainless steel cages and were allowed at least 24 h for recovery from surgery. Food and water were provided ad libitum. On the day of the experiment, the heart rate of each rat was monitored with plethesmograph (Buffington Clinical Devices) and the data recorded on a Physiocribe II recorder (Stoetling Co.). One hour was allowed as an equilibration period before any drugs were administered. Drugs were dissolved in normal saline as 0.3% solutions and were administered iv at a dose of 6 mg/kg. The resting heart rate was then recorded 1, 3, 5, 10, and 15 min following injection. Isoprenaline (isoproterenol bitartrate, Sigma Co.) was then administered subcutaneously at a dose of 50 μg/kg, and the heart rate was recorded at 3, 5, 10, 15, 20, 30, 45, and 60 min after administration. A control group of seven animals was given saline solution iv and then treated in the same manner as the drug-treated animals. Significance of the difference between the effect of saline solution and test compounds on resting heart rate and on isoprenaline-induced tachycardia was analyzed by using the Student's t-test. Volumes are given as means ± SE of the mean.

Duration of Action of Compounds 3d, 3g, and 4 on Heart Rate and on Isoprenaline-Induced Tachycardia. Male Sprague–Dawley rats were prepared as described above. Test compounds were dissolved in normal saline and were administered at a dose of 6 mg/kg. Animals received the test compound either 30, 60, 90, or 180 min prior to an sc dose of isoprenaline (50 μg/kg). Heart rate was recorded at 0, 3, 5, 10, 15, 20, 30, 45, and 60 minutes

following administration of isoprenaline. A control group of five animals received iv saline and was treated in the same manner as drug-treated groups. Statistical analysis was performed as described above.

Registry No. **3a**, 101479-72-5; **3a** (oxalate), 114652-88-9; **3b**, 101479-70-3; **3b** (oxalate), 114652-89-0; **3c**, 114652-90-3; **3c** (oxalate), 114652-91-4; **3d**, 114652-92-5; **3d** (oxalate), 114652-93-6; **3f**, 113296-04-1; **3f** (oxalate), 114652-94-7; **3g**, 114652-95-8; **3g** (oxalate), 114652-96-9; **5**, 156-38-7; **6**, 17138-28-2; **7**, 114652-97-0; **8**, 114652-98-1; **9a**, 114652-99-2; **9b**, 114653-00-8; **9c**, 114653-01-9; **9d**, 114653-02-0; **9e**, 114653-03-1; **9f**, 114653-04-2; **10a**, 114653-05-3; **10b**, 114653-06-4; **10c**, 114653-07-5; **10d**, 114653-08-6; **10e**, 114653-09-7; **10f**, 114653-10-0; **11a**, 114653-11-1; **11b**, 114653-12-2; **11c**, 114653-13-3; **11d**, 114653-14-4; **11f**, 114653-15-5; **11g**, 114653-16-6; **12**, 6975-91-3; **13**, 83053-47-8; **14**, 114653-17-7; adamantylmethanol, 770-71-8; adamantylethanol, 6240-11-5; (+)-isopinocampheol, 24041-60-9; 1*s*-*endo*-borneol, 464-45-9; *exo*-norborneol, 497-37-0; *endo*-norborneol, 497-36-9; cyclohexanol, 108-93-0; chloroacetyl chloride, 79-04-9.

Effects of Sulfur-Containing Analogues of Stearic Acid on Growth and Fatty Acid Biosynthesis in the Protozoan *Crithidia fasciculata*

Mohammad D. Rahman, David L. Ziering, Sara J. Mannarelli, Karen L. Swartz, Ded-Shih Huang, and Robert A. Pascal, Jr.*

Department of Chemistry, Princeton University, Princeton, New Jersey 08544. *Received August 31, 1987*

A variety of analogues of stearic acid in which one of the methylene groups was replaced by a sulfur atom were examined as inhibitors of growth and fatty acid biosynthesis in the trypanosomatid protozoan *Crithidia fasciculata*. The 8-, 9-, 10-, and 11-thiastearic acids were found to suppress the synthesis of the cyclopropane-containing fatty acid dihydrosterculic acid (9,10-methyleneoctadecanoic acid) at micromolar concentrations in the growth medium, and all but the 9-thiastearate were found to inhibit the growth of the protozoa at comparable concentrations. The most potent inhibitor, 8-thiastearic acid (I_{50} for growth = 0.8 μM; I_{50} dihydrosterculate synthesis = 0.4 μM), was also observed to inhibit the synthesis of γ-linolenic acid at a similar concentration. The sulfoxide derivatives of the 9- and 10-thiastearates were found to have little effect on growth or fatty acid synthesis, and several long-chain amides of 3-amino-1,2-propanediol were found to have effects similar to those of the fatty acids from which they were derived.

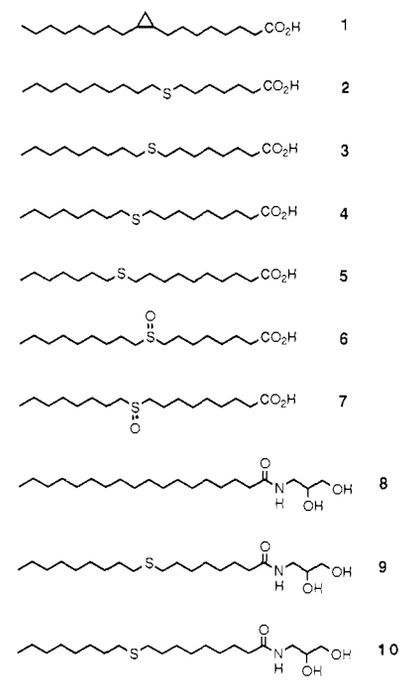
Dihydrosterculic acid (9,10-methyleneoctadecanoic acid, **1**), a cyclopropane fatty acid widely distributed in bacteria and plants,[1] is also a major constituent of the phospholipids of many trypanosomatid flagellates, including several pathogenic species of *Leishmania*.[2,3] The presence of compound **1** is one of the "biochemical peculiarities" of these protozoa[4]—at least with respect to mammals, which do not synthesize or require cyclopropane fatty acids. If dihydrosterculic acid is not merely present in the protozoa but in fact *required* for normal growth, then specific inhibitors of the biosynthesis of **1** might be useful antiparasitic agents.

We recently observed that 10-thiastearic acid (**4**, Chart I) is a potent inhibitor of dihydrosterculic acid biosynthesis in *Crithidia fasciculata*.[5] In addition, we found that the growth of the protozoa is inhibited at concentrations comparable to those required for the inhibition of cyclopropane synthesis, suggesting that dihydrosterculic acid is required for the normal growth of these organisms. In this paper we report additional studies with 10-thiastearic acid and examine the effects of a variety of other sulfur-containing analogues of stearic acid on growth and fatty acid biosynthesis in *C. fasciculata*.

Chemistry

The syntheses of the free fatty acids in which C-9 or C-10 is replaced by a sulfur-containing functional group

Chart I



(**3**, **4**, **6**, and **7**) were described previously,[6] and the 8- and 11-substituted acids (**2** and **5**) were prepared by using similar methodology.

The syntheses of the long-chain amides **8**–**10** are somewhat unusual. We first attempted to prepare these *N*-(2,3-dihydroxypropyl) amides by condensation of the appropriate fatty acyl chlorides with 3-amino-1,2-

(1) Law, J. H. *Acc. Chem. Res.* **1971**, *4*, 199–203 and references cited therein.
(2) Meyer, H.; Holz, G. G., Jr. *J. Biol. Chem.* **1966**, *241*, 5000–5007.
(3) (a) Fish, W. R.; Holz, G. G., Jr.; Beach, D. H.; Owen, E.; Anekwe, G. E. *Mol. Biochem. Parasitol.* **1981**, *3*, 103–115. (b) Holz, G. G., Jr. In *Leishmaniasis*; Chang, K.-P., Bray, R. S., Eds.; Elsevier: New York, 1985.
(4) Opperdoes, F. R. *Br. Med. Bull.* **1985**, *41*, 130–136.
(5) Pascal, R. A., Jr.; Mannarelli, S. J.; Ziering, D. L. *J. Biol. Chem.* **1986**, *261*, 12441–12443.
(6) Pascal, R. A., Jr.; Ziering, D. L. *J. Lipid Res.* **1986**, *27*, 221–224.
(7) Taylor, F. R.; Grogan, D. W.; Cronan, J. E., Jr. *Meth. Enzymol.* **1981**, *71*, 133–139.

0022-2623/88/1831-1656$01.50/0