(11a). A solution of 10 (700 mg, 1.04 mmol) in MeOH (30 mL) in the presence of 5% Pd–C (200 mg) was stirred for 15 min under a stream of H_2. The catalyst was filtered off and washed with AcOEt and $CHCl_3$. The filtrate and washings were combined and concentrated to give 11a (686 mg, 97.7%) as a pale pink solid: mp 172–178 °C dec; ^{1}H NMR ($CDCl_3$) 7.28–7.38 (10 H, m, Ph_2), 6.99 (1 H, s, O*H*), 6.91 (1 H, s, C*H*Ph_2), 6.83 (1 H, s, O*H*), 4.25 (1 H, t, J = 3.6 Hz, 7-H), 4.07 (1 H, m, 5-H), 3.84 (1 H, dd, J = 11.0, 3.3 Hz, 14-H), 3.76 (1 H, br, 14-H), 3.57 (1 H, br s, 1-H), 3.51 (1 H, m, 4-H), 3.31–3.39 (2 H, m, SC*H*Me_2 × 2), 3.29 (1 H, dd, J = 10.3, 5.9 Hz, 2-H), 3.10 (1 H, m, 12a-H), 3.07 (1 H, dd, J = 15.8, 2.8 Hz, 12-H), 2.69 (1 H, m, 3-H), 2.45 (1 H, dd, J = 15.8, 11.4 Hz, 12-H), 2.19 (3 H, br s, NCH_3), 2.17 (1 H, m, 3-H), 1.22–1.29 (12 H, m, SCH(CH_3)$_2$ × 2) ppm; IR (KBr) 3426, 3398, 2966, 2924, 2866, 1724, 1593, 1495, 1458, 1447, 1406, 1387, 1363, 1327, 1274, 1223, 1151, 1089, 1065, 1052, 1027, 995, 929, 873, 857, 754, 736, 702 cm^{-1}; EIMS m/z 673 (M^+), 643, 354, 167. Anal. ($C_{37}H_{43}N_3O_5S_2 \cdot 2H_2O$) C, H, N.

Diphenylmethyl 9,10-Bis(isopropylthio)-5-cyano-8,11-dimethoxy-7-(hydroxymethyl)-13-methyl-1,2,3,4,5,7,12,12a-octahydro-1,4-iminoazepino[1,2-*b*]isoquinoline-2-carboxylate (11b). To a solution of 11a (600 mg, 1.04 mmol) in DMF (30 mL) was added K_2CO_3 (148 mg, 1.07 mmol). MeI (0.26 mL, 4.2 mmol) and $NaBH_4$ (11 mg) were added portionwise followed by 25 h of stirring. The reaction mixture was concentrated and partitioned between AcOEt and H_2O. The organic layer was separated and washed with brine, dried, and concentrated. The residue was subjected to chromatography (SiO_2, 70 mL, *n*-hexane–AcOEt 4:1–3:1) to give 11b (414 mg, 66.2%): ^{1}H NMR ($CDCl_3$) 7.30 (10 H, m, Ph_2), 6.87 (1 H, s, C*H*Ph_2), 4.00–4.24 (2 H, m, 5- and 7-H), 3.84 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.32–3.76 (6 H, m), 3.26 (1 H, dd, J = 9, 6 Hz, 2-H), 2.84–3.16 (2 H, m), 2.24–2.80 (2 H, m), 2.13 (3 H, s, NCH_3), 1.98 (1 H, m, 3-H), 1.12–1.36 (12 H, m, SCH(CH_3)$_2$ × 2) ppm; SIMS m/z 702 (M + 1)$^+$, 675 (M + 1 – HCN)$^+$, 509.

9,10-Bis(isopropylthio)-5-cyano-8,11-dimethoxy-7-(hydroxymethyl)-13-methyl-1,2,3,4,5,7,12,12a-octahydro-1,4-iminoazepino[1,2-*b*]isoquinoline-2-carboxylic Acid (12a). With the same procedure as that for 7a–d, 11b (385 mg) provided 12a (246 mg, 83.7%): SIMS m/z 536 (M + 1)$^+$, 522, 509 (M + 1 – HCN)$^+$, 495.

Conversion of 12a to Oxazolidine Form 12b. With the same procedure as that for 5a–d, 12a (90 mg) gave 12b (80.1 mg, 93.7%): ^{1}H NMR (CD_3OD) 4.56 (1 H, d, J = 3.1 Hz, 5-H), 4.45 (1 H, dd, J = 7.2, 3.1 Hz, 7-H), 4.14 (1 H, br s, 1-H), 4.12 (1 H, m, 4-H), 3.83 (3 H, s, OCH_3), 3.76 (3 H, s, OCH_3), 3.61–3.71 (2 H, m, 14-H_2), 3.34–3.41 (3 H, m, 1-H and SC*H*Me_2 × 2), 3.25 (1 H, m, 12a-H), 3.06 (1 H, dd, J = 15.2, 2.5 Hz, 12-H), 2.80 (3 H, s, NCH_3), 2.64 (1 H, m, 3-H), 2.54 (1 H, dd, J = 15.2, 12.0 Hz, 12-H), 2.47 (1 H, dd, J = 13.7, 10.4 Hz, 3-H), 1.15–1.23 (12 H, m, SCH(CH_3)$_2$ × 2) ppm; SIMS m/z 509 (M + 1)$^+$, 495, 467.

Biological Studies. HeLa S_3 cells (5 × 10^4) were seeded in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing of 10% fetal bovine serum (Grand Island Biological Co.) and 0.06 mg/mL of kanamycin. Graded concentrations of drugs, appropriately diluted with growth medium, were added 24 h after the cells were seeded. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 72 h of drug exposure, the monolayer cells were washed with phosphate-buffered salts solution (Flow Laboratories) and incubated with 0.05% trypsin (Difco Laboratories, Detroit, MI) and 0.02% EDTA (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan). The cells were counted with a Toa Micro-Cell counter (Toa Medical Electronics Co., Ltd., Kobe, Japan) and the IC_{50} value (drug concentration required for 50% inhibition of the cell growth) was determined.

Lymphocytic leukemia P388 (1 × 10^6) cells were implanted intraperitoneally (ip) into $CD2F_1$ mice (about 22 g weight) divided into groups each consisting of five test mice. Administration of drugs was started the day after tumor implantation. Antitumor efficacy was expressed as an increased life span (ILS), calculated $(T/C - 1) \times 100$, where T and C are median survival times of treated and control mice.

Sarcoma 180 (5 × 10^6 cells/mouse) was inoculated subcutaneously (sc) at the axillary region in ddY mice divided into groups each consisting of five test mice. Drugs were administered intravenously (iv) starting the day after tumor inoculation. Antitumor efficacy was expressed as T/C, where T and C are mean tumor volume of treated and control mice. Tumor volume was calculated by using the formula for a prolate ellipsoid

$$\text{tumor volume} = \text{L (mm)} \times W^2 \text{ (mm)}/2$$

in which L is the length of the major axis and W is the length of the minor axis.[14]

BALB/c-nu/nu mice were administered with a tumor fragment equivalent to 8 mm^3 of MX-1 (human mammary carcinoma), Co-3 (human colon carcinoma), LC-06 (human lung carcinoma) or St-4 (human gastric carcinoma) tumor passed in nude mouse. When tumor volume reached 100–300 mm^3, the mice were pair matched in groups of five each and the drug was administered intravenously. Antitumor efficacy was expressed as T/C, as described for sarcoma 180.

Supplementary Material Available: Listings of complete analytical data and physicochemical data (^{1}H and ^{13}C NMR, IR and mass spectrum) of new compounds except for those described in the paper (7 pages). Ordering information is given on any current masthead page.

(14) Geran, R.; Greenberg, N. H.; MacDonald, M. M.; Schumaker, A. M.; Abbott, B. J. *Cancer Chemother. Rep.* **1972**, *3*, 1.

Persistent Binding of Fatty Acyl Derivatives of Naltrexamine to Opioid Receptors

P. S. Portoghese,* A. Garzon-Aburbeh, and D. L. Larson

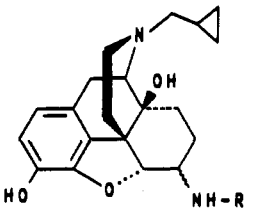
Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.
Received November 30, 1990

A series of fatty acid derivatives of naltrexamine and naltrexhydrazine were synthesized and evaluated for their persistent binding to opioid receptors. Members of this series were found to require greater than five washes for removal from mouse brain membranes when the fatty acyl chain was saturated. The presence of unsaturation in the fatty acyl groups enhanced the persistent binding. In this regard the persistent binding increased as a function of the number of double bonds, with the unsaturated congeners requiring greater than 10 washes for removal of the ligand from the membranes. The results of this study are consistent with the apparently important role of polyunsaturated fatty acids in the binding of ligands to opioid receptors.

Typically, common opioid ligands such as morphine or naloxone readily dissociate from opioid receptors and are nearly completely removed from brain membranes after a single wash. A notable exception to this behavior was originally noted for opiate hydrazones and azines (e.g., naloxonazine), which required at least two washings for

0022-2623/91/1834-1966$02.50/0 © 1991 American Chemical Society

Table I. Fatty Acid Amide and Hydrazides Derivatives



compd no.	R	isomer	% yield	mp, °C	*m/z* (M^+)	formula[a]
4a	$COCH_3$	6α	83	258–260	385 (m + 1)	$C_{22}H_{28}N_2O_4$
4b	$COCH_3$	6β	85	>270	385 (m + 1)	$C_{22}H_{28}N_2O_4 \cdot HCl \cdot H_2O$
5a	$CO(CH_2)_{14}CH_3$	6α	69	63–64	580	$C_{36}H_{56}N_2O_4$
5b	$CO(CH_2)_{14}CH_3$	6β	96	65–67	580	$C_{36}H_{56}N_2O_4$
6a	$CO(CH_2)_{16}CH_3$	6α	80	74–77	608	$C_{38}H_{60}N_2O_4$
6b	$CO(CH_2)_{16}CH_3$	6β	90	79–81	608	$C_{38}H_{60}N_2O_4$
7a	$CO(CH_2)_7CH{=}CH(CH_2)_7CH_3$	6α	62	oil	606	$C_{38}H_{58}N_2O_4$
7b	$CO(CH_2)_7CH{=}CH(CH_2)_7CH_3$	6β	28	oil	606	$C_{38}H_{58}N_2O_4$
8a	$CO(CH_2)_7(CH{=}CHCH_2)_2(CH_2)_3CH_3$	6α	44	oil	604	$C_{38}H_{56}N_2O_4$
8b	$CO(CH_2)_7(CH{=}CHCH_2)_2(CH_2)_3CH_3$	6β	38	oil	604	$C_{38}H_{56}N_2O_4$
9a	$CO(CH_2)_7(CH{=}CHCH_2)_3CH_3$	6α	21	oil	602	$C_{38}H_{54}N_2O_4$
9b	$CO(CH_2)_7(CH{=}CHCH_2)_3CH_3$	6β	27	oil	602	$C_{38}H_{54}N_2O_4$
10	$NHCO(CH_2)_{14}CH_3$	6β	64	64–67	595	$C_{36}H_{57}N_3O_4$
11	$NHCO(CH_2)_{16}CH_3$	6β	72	74–76	623	$C_{38}H_{61}N_3O_4$
12	$NHCO(CH_2)_7(CH{=}CHCH_2)_3CH_3$	6β	29	oil	617	$C_{38}H_{55}N_3O_4$

[a] All compounds were within 0.4% of the calculated values for C, H, N analyses for the formulas listed above.

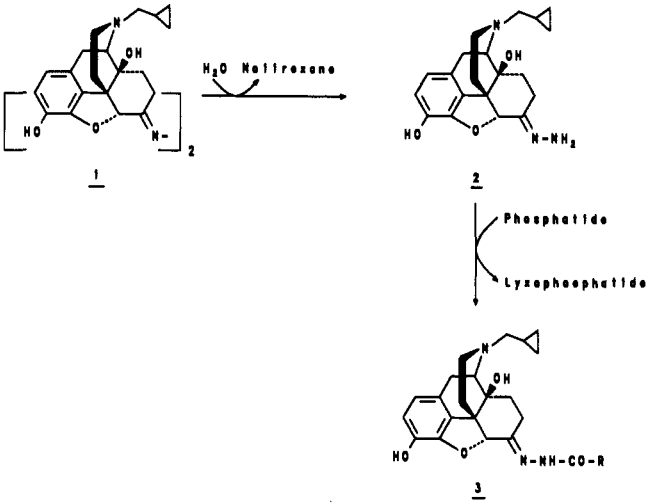
Table II. Binding of Fatty Acyl Derivatives of Naltrexamine and Naltrexhydrazine to Mouse Brain Membranes[a]

compd no.	no. of washes			prewashed
	3	5	10	
4a	2 ± 2			
4b	4 ± 2			
5a	85 ± 4	35 ± 3	3 ± 3	24 ± 4
5b	93 ± 5	58 ± 6	2 ± 2	38 ± 4
6a	77 ± 6	42 ± 5	6 ± 2	18 ± 3
6b	89 ± 6	48 ± 4	2 ± 3	34 ± 4
7a	98 ± 7	54 ± 3	21 ± 3	42 ± 4
7b	95 ± 6	93 ± 5	32 ± 4	64 ± 5
8a	93 ± 6	93 ± 4	46 ± 5	73 ± 5
8b	94 ± 5	96 ± 5	53 ± 3	84 ± 6
9a	94 ± 6	89 ± 6	84 ± 3	69 ± 4
9b	92 ± 7	95 ± 5	86 ± 5	85 ± 3
10	33 ± 5	2 ± 3		
11	38 ± 6	4 ± 4		
12	93 ± 9	46 ± 5	38 ± 3	
1	35 ± 3	−1 ± 4	2 ± 3	

[a] Expressed as a percentage of apparent receptor occupancy by the ligand as determined from the difference in specific binding of [^{3}H]dihydromorphine obtained before and after the wash procedure (see Experimental Section). The percent binding of ligands to previously washed membranes was determined in the same way. The values represent the mean ± SD of triplicate experiments.

removal.[1] Subsequently, it was reported that opiate hydrazones that contain hydrophobic residues attached to the hydrazine moiety also exhibited persistent opioid receptor binding to brain membranes.[2]

Recently, we reported[3] that the persistent opioid receptor binding of hydrazine-containing naltrexone derivatives (e.g., naltrexonazine, **1**) is related to the conversion of such ligands to fatty acylnaltrexazones **3**. The fatty acyl group was found to be derived from membrane phosphatides. Evidence was presented that suggested that naltrexonazine was hydrolyzed in the membrane to naltrexazone (**2**), and that this intermediate subsequently reacted with the ester carbonyl of a receptor-associated phosphatide to afford fatty acylnaltrexazone **3**. We proposed that the wash-resistant property of naltrexonazine may be due to localization of its derived fatty acylhydrazone derivative into the lipid bilayer of the membrane as a consequence of hydrophobic bonding. This might permit ready access to opioid receptors by lateral diffusion of the ligand. The fact that several synthetically derived fatty acylnaltrexazones exhibited a high degree of persistent binding to brain membranes was consistent with this idea.[3]



1 2 3

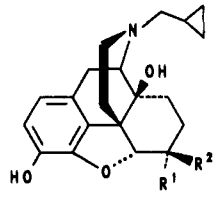
We have investigated this mechanism further by synthesizing a series of amides containing fatty acyl groups of different length and varying degrees of unsaturation. The results of binding studies in the present study provide additional support that the presence of an appropriate fatty acyl chain contributes to the persistent binding to opioid receptors.

Chemistry

The amides **4–9** were synthesized from 6α- or 6β-naltrexamine[4] (**13a,b**) and the corresponding acid (stearic,

(1) Hahn, E. F.; Carroll-Buatti, M.; Pasternak, G. W. *J. Neurosci.* **1982**, *2*, 572.
(2) Hahn, E. F.; Nishimura, S.; Goodman, R. R.; Pasternak, G. W. *J. Pharmacol. Exp. Ther.* **1985**, *235*, 839.
(3) Garzon-Aburbeh, A.; Lipkowski, A. W.; Larson, D. L.; Portoghese, P. S. *Neurochem. Int.* **1989**, *15*, 207.

palmitic, acetic, oleic, linoleic, and linolenic acids) using dicyclohexylcarbodiimide (DCC) in dichloromethane. Similarly, the hydrazide derivatives were obtained by reacting 6β-naltrexhydrazine[3] (14) with palmitic, stearic, or linoleic acids in the presence of DCC in dimethylformamide.



13a $R^1 = NH_2$, $R^2 = H$

13b $R^1 = H$, $R^2 = NH_2$

14 $R^1 = H$, $R^2 = NH\text{-}NH_2$

Receptor Binding

Binding assays were performed on the target compounds (4–12) by a modification of previously described procedures[3,5] using mouse brain membranes. The compounds were incubated with the membranes for 30 min at 25 °C followed by 3, 5, or 10 washes. In order to measure the receptor occupancy, the washed membranes in each determination were incubated with 2 nM [^{3}H]dihydromorphine and the specific binding was compared with that of a control experiment treated identically but without the addition of target compound. The ratio was expressed as the percentage of persistent binding.

With the exception of the acetamides (4a, 4b) all of the target compounds exhibited persistent binding (Table II). The saturated fatty acyl derivatives were more easily washed from the membranes than those that contain double bonds. The 6α isomers appear to be removed from the membranes more easily than the corresponding β epimers. The fatty acyl hydrazides (10–12) bound less persistently than the corresponding amides.

Discussion

We have previously reported[3] that the fatty acylhydrazone product derived from naltrexonazine is responsible for the apparent persistent binding of the parent azine. Also, it was demonstrated that the source of the fatty acyl group was a phosphatide pool that could be removed upon repeated washing. This suggested that the phosphatides involved in the acyl transfer reaction were not an integral part of the lipid bilayer. In view of the evidence for the involvement of lipids in opioid receptor operation,[5-11] it is conceivable that this pool of lipid is closely associated with the opioid receptor system.

It was proposed[3] that the membrane may act as a reservoir for the long-chain fatty acid moiety of the fatty acylhydrazone product. Thus, we viewed persistent binding to be a consequence of the slow removal of fatty acylhydrazone from the membrane lipid bilayer upon repeated washing. In this regard, it seemed likely that the trapped acylhydrazone may have access to the receptor by lateral diffusion in the membrane bilayer.

The present study supports our earlier observations and conclusions in that it demonstrates that a long-chain fatty acyl group is a requirement for persistent binding. Interestingly, the amides require a greater number of washes than the corresponding acylhydrazones to obtain the same percent reduction of specific binding. On the other hand, hydrazides 10–12 have a wash-out profile that resembles that of the acylhydrazones.

Significantly, the persistent binding increases as the degree of unsaturation increases in the fatty acyl group. This occurs regardless of the nature of the derivative, whether it be amide, hydrazide, or acylhydrazone. In the amide series this is more apparent after 10 washes, where the percent specific binding remaining ranged from 2% to 86% as the number of double bonds is increased from 0 to 3 in the acyl chain. The hydrazides exhibit less persistent binding than the corresponding amides and consequently this effect is more noticeable after five washes. As a point of reference, naltrexonazine 1, which is known to be converted to the acylhydrazone after receptor binding, exhibited only 35% specific binding after three washes and no significant binding after five washes.

It is noteworthy that all of the fatty acyl derivatives 5–12 exhibited persistent binding on the previously washed membrane preparation. This is in contrast to naltrexonazine (1), which was removed by washing after it was bound under identical conditions. These results indicate that the unique pool of lipid that donates the fatty acyl group to intermediate 2 in the conversion of 1 to 3, is not required for the persistent binding of the target compounds 5–12 because they already contain a fatty acyl group.

The fact that unsaturated fatty acyl groups attached to opiate structures generally enhance persistent binding may be a consequence of more facile intercalation of the fatty acyl derivative into the lipid bilayer and/or to interactions of the fatty acyl derivatives with opioid receptor systems. With regard to the latter possibility, it is significant that there is substantial evidence for the involvement of lipids in opioid receptor binding.[6-10] In fact, polyunsaturated fatty acids have been reported to confer higher affinity to the binding of opioid ligands.[11]

Experimental Section

Melting points are determined with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by MHW Laboratories and were within ±0.4% of the theoretical value. CI-MS (NH_3 gas) were obtained on a Finnigan 4000 instrument. HPLC separation was performed on a Beckman model 110-A with a preparative silica gel column (DYNAMAX, 21.4 mm i.d. × 25 cm, 8 μm) using ethyl acetate–hexane–NH_4OH (65:35:1).

6-(Acylamido)-17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinans (4–9). α- or β-naltrexamine 13 (100 mg, 0.3 mmol) was dissolved in CH_2Cl_2, and to this solution was added successively (dimethylamino)pyridine (DMAP) (10 mg), the fatty acid (0.32 mmol), and dicyclohexylcarbodiimide (DCC) (120 mg, 0.48 mmol). After 3 h, the precipitate was removed by filtration, and the filtrate was partitioned between aqueous $NaHCO_3$ (10 mL) and ethyl acetate. The organic layer was washed with aqueous $NaHCO_3$ and H_2O and dried ($NaSO_4$), and the solvent was removed in vacuo. The compounds were purified by column chromatography using hexane–ether (70:30). All crystalline compounds were crystallized from acetone.

(4) Jiang, J. B.; Hanson, R. N.; Portoghese, P. S.; Takemori, A. E. *J. Med. Chem.* **1977**, *20*, 1100.
(5) Pasternak, G. W.; Wilson, H. A.; Snyder, S. H. *Molec. Pharmacol.* **1975**, *11*, 340.
(6) Abood, L. G.; Salem, N.; McNeil, M.; Butler, M. *Biochem. Biophys. Acta* **1978**, *530*, 35.
(7) Dunlap, C. E. III; Leslie, F. M.; Rado, M.; Cox, B. M. *Molec. Pharmacol.* **1979**, *16*, 105.
(8) Loh, H. H.; Law, P. Y. *Annu. Rev. Pharmacol.* **1980**, *20*, 201.
(9) Garzon, J.; Jen, M. F.; Lee, N. M. *Biochem. Pharmacol.* **1983**, *32*, 1523.
(10) Farahbakhsh, Z. T.; Beamer, D. W.; Lee, N. M.; Loh, H. *J. Neurochem.* **1986**, *46*, 953.
(11) Haseqawa, J. I.; Loh, H. H.; Lee, N. M. *J. Neurochem.* **1987**, *49*, 1007.

17-(Cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxy-6-hydrazidomorphinans (10–12). To a solution containing 14 (50 mg, 0.14 mmol) and fatty acid (0.15 mmol) in dimethylformamide (5 mL) was added DMAP (1.7 mg, 0.15 mmol) and DCC (37 mg, 0.18 mmol). After 3 h at 23 °C, the mixture was filtered, and the filtrate was chromatographed on a silica gel column using ethyl acetate–hexane (60:30). The common feature of the ^{1}H NMR for the β-hydrazide derivatives was the doublet corresponding to C5-H at δ 4.5 (J(5,6) = 7.43–7.62 Hz).

Binding Assay. Membrane Preparation. Male mice (20–25 g) were decapitated and the brains minus the cerebellum were removed and frozen immediately on dry ice. They were weighed and homogenized in 10 volumes of 0.32 M sucrose in a Duall tissue grinder (Kontes) homogenizer with the pestle driven by a motor at about 2000 rpm. The homogenate was centrifuged at 1000g in 16-mL polypropylene tubes in a Superspeed centrifuge for 10 min. The supernatant was centrifuged for 30 min at 17000g. The pellet obtained was osmotically shocked with three volumes of ice-cold distilled water and centrifuged for 20 min at 12000g. The supernatant was carefully decanted and diluted 1:1 with pH 7.4 Hepes buffer (NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.2 mM; Hepes, 25 mM) and centrifuged for 30 min at 40000g. This final pellet was resuspended in Hepes buffer, diluted to four volumes of the initial wet weight, and either used immediately or saved at −80 °C for further experiments.

Binding Procedures. Membrane homogenate (0.4 mL) and 0.025 mL of unlabeled ligand (50 nM) in distilled water were added to a 1.5-mL polypropylene microfuge tube (Kontes), incubated for 30 min at 25 °C, and centrifuged in a Fisher Microcentrifuge at 11 000 rpm for 5 min. The capped tubes then were inverted and spun at 800g for 30 s on a rotating wheel to separate the pellet and supernatant. For each wash cycle, the membrane was resuspended in 30 μL of fresh Hepes buffer with the aid of a special pestle (Kontes), diluted with an additional 1 mL of buffer, incubated for 10 min at 37 °C, and recentrifuged. To estimate the remaining apparent receptor occupancy by the ligand, the washed sample was resuspended in a mixture of 200 μL of buffer containing [^{3}H]dihydromorphine (67 Ci/mmol, final concentration 2 nM) and 25 μL of either buffer alone or buffer with unlabeled naltrexone (final concentration 1 μM). This mixture was incubated for 20 min at 25 °C and centrifuged, and the supernatant was removed as described above. The tips of the tubes containing the membrane pellet were cut off and transferred to a scintillation vial. The pellet was emulsified in Biofluor and the radioactivity counted with a Beckman LS 6800 liquid scintillation counter at an efficiency of about 50%. The apparent receptor occupancy after each wash cycle was determined from the change in the specific binding of [^{3}H]dihydromorphine to membranes treated with fatty acyl ligand as compared to specific binding of membranes in a control experiment without ligand present. Specific binding is defined as the difference in cpm for membranes incubated with [^{3}H]dihydromorphine in the presence and in the absence of naltrexone.

Acknowledgment. This research was supported by the National Institute on Drug Abuse.

Registry No. 4a, 134031-51-9; **4b**, 134031-59-7; **5a**, 134107-40-7; **5b**, 117250-89-2; **6a**, 134031-52-0; **6b**, 134031-60-0; **7a**, 134031-53-1; **7b**, 134054-98-1; **8a**, 134031-54-2; **8b**, 134107-41-8; **9a**, 134031-55-3; **9b**, 134107-42-9; **10**, 134031-56-4; **11**, 134031-57-5; **12**, 134031-58-6; **13a**, 84774-95-8; **13b**, 67025-97-2; **14**, 124206-70-8; acetic acid, 64-19-7; oleic acid, 112-80-1; linolenic acid, 463-40-1; stearic acid, 57-11-4; palmitic acid, 57-10-3; linoleic acid, 60-33-3.

Lipopeptides Containing 2-(Palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic Acid: Synthesis, Stereospecific Stimulation of B-Lymphocytes and Macrophages, and Adjuvanticity in Vivo and in Vitro

Jörg Metzger,[†] Günther Jung,*[,†] Wolfgang G. Bessler,[‡] Petra Hoffmann,[‡] Marianne Strecker,[‡] Albrecht Lieberknecht,[§] and Ulrich Schmidt[§]

Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen, FRG, Institute of Immunobiology, University of Freiburg, Stefan-Meier-Strasse 8, D-7800 Freiburg, FRG, and Institute of Organic Chemistry, Biochemistry and Isotope Research, University of Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart 80, FRG.
Received March 19, 1990

Lipopeptides, carrying the N-terminal lipoamino acid 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid (Pam_3Adh-OH, **1**), were obtained by solid-phase synthesis and by synthesis in solution. 2-Amino-6,7-dihydroxyheptanoic acid (Adh) can be regarded as a methylene analogue of *S*-glycerylcysteine, the N-terminal amino acid of lipoprotein from the outer cell membrane of *Escherichia coli* (a methylene group substitutes for the sulfur atom). The lipopeptides Pam_3Adh-Ser-Ser-Asn-Ala **2a–d**, in which the four possible stereoisomers of Pam_3Adh-OH (2*S*,6*S*)-**1** (**1a**), (2*S*,6*R*)-**1** (**1b**), (2*R*,6*S*)-**1** (**1c**), and (2*R*,6*R*)-**1** (**1d**) are linked to the naturally occurring sequence Ser-Ser-Asn-Ala of the N-terminus of lipoprotein, and also Pam_3Adh-Ser-$(Lys)_4$ ((2*S*,6*S*)-**3**), with a peptide part rendering the molecule water soluble, were capable of stimulating murine splenocytes polyclonally in vitro, as determined in a proliferation assay and in a hemolytic plaque assay against trinitrophenylated sheep erythrocytes. The diastereomers (2*S*,6*S*)-**2** and (2*R*,6*S*)-**2** with *S*-configurated C-6 were more active than the diastereomers (2*S*,6*R*)-**2** and (2*R*,6*R*)-**2** with *R*-configurated C-6; a change of the configuration at C-2 had less effect on the stimulatory activity. (2*S*,6*S*)-**2** and (2*S*,6*S*)-**3** are potent immunoadjuvants. A significantly enhanced primary immune response against trinitrophenylated sheep erythrocytes was obtained in vitro at lipopeptide concentrations of about 5 μg/mL and an immunization dose of 10^7 sheep erythrocytes/mL. Balb/c mice, which were immunized with a mixture of ovalbumin and (2*S*,6*S*)-**2** or (2*S*,6*S*)-**3**, respectively, had a substantially higher antiovalbumin titer 28 days after immunization than mice which had received ovalbumin, (2*S*,6*S*)-**2** or (2*S*,6*S*)-**3** alone. Finally, the novel lipopeptides constitute potent macrophage activators: (2*S*,6*S*)-**3** was able to induce tumor cytotoxicity against the tumor cell line L929 in bone marrow derived macrophages.

Synthetic lipopeptides derived from the N-terminus of lipoprotein from the outer cell membrane of *Escherichia coli* are macrophage activators[1,2] and B-lymphocyte stimulants.[3-5] Conjugates in which these lipopeptides are covalently coupled to antigens or mixtures of lipopeptides and antigens induce antigen-specific antibodies in vivo and

[†] University of Tübingen.
[‡] University of Freiburg.
[§] University of Stuttgart.

(1) Hoffmann, P.; Heinle, S.; Schade, U. F.; Loppnow, H.; Ulmer, A. J.; Flad, H.-D.; Jung, G.; Bessler, W. G. *Immunobiology* **1988**, *177*, 158.
(2) Hoffmann, P.; Wiesmüller, K.-H.; Metzger, J.; Jung, G.; Bessler, W. G. *Biol. Chem. Hoppe-Seyler* **1989**, *370*, 575.

0022-2623/91/1834-1969$02.50/0 © 1991 American Chemical Society