Parallel Synthesis of a Lipopeptide Library by Hydrazone-Based Chemical Ligation

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α-Melanocyte-stimulating hormone (α-MSH) is an endogeneous linear tridecapeptide with potential application for the modulation of skin tanning. To evaluate the interest of introducing a lipid moiety onto this peptide, we developed an efficient chemoselective parallel method to prepare a large series of analogues of α-melanocortin with high purity, varying the nature or the relative position of the lipid moiety. Two sets of building blocks containing lipidic α-oxo-aldehydes or α-hydrazinoacetyl peptides were combined to obtain a 102-membered library of amphiphilic α-MSH analogues. This library was pharmacologically tested at 1×10^{-7} M for the ability to induce AMPc production in M4Be melanoma cell line after stimulation of the human melanocortin MC1 receptor. Among theses lipopeptides, 84 compounds exhibited an AMPc induction higher than Melitane, a patented α-MSH agonist. These results provide strong evidence of the interest of introduction of a lipid tail for the pharmacomodulation of bioactive peptides.

Introduction

α-Melanocyte-stimulating Hormone (α-MSH) is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) synthesized in pituitary[1] and skin.[2] This hormone binds to four melanocortin receptor subtypes. In this work, we focused our attention on MC1 receptor subtype (MC1-R), which plays an important role in pigmentation and inflammation.[3] MC1-R is expressed in pituitary, skin (melanoma cells, melanocytes, and skin glands), and anti-inflammatory cells. Therefore, analogues of α-MSH binding to MC1-R may have three major applications. Agonists could be used as anti-inflammatory or skin tanning products, whereas antagonists have potential applications as skin-bleaching agents.

The first studies of structure–activity relationship identified the sequence His-Phe-Arg-Trp as the minimal active sequence.[4–6] During the last few years, analogues of α-MSH have been synthesized and characterized.[7] Introduction of modifications within the peptidic sequence of α-MSH included several truncations, cyclization, substitutions with other amino acids, N- and C-terminal modifications, and more recently, introduction of β-turn mimic.[8] Moreover, it has been clearly shown by our group[9] and others[10] that introduction of lipidic chains at the C terminus significantly enhanced the pharmacological properties of α-MSH analogues.

To evaluate the interest of introducing a lipid moiety onto α-MSH analogues and their ability to modulate the activity of MC1-R, we modified active peptides by different fatty acids. Synthetic lipopeptides are usually obtained by introduction of the acyl moiety onto the growing peptide chain during solid-phase synthesis. The separation of the target compound from impurities by RP-HPLC is often complicated by the amphiphilic properties of lipopeptides and results in low overall yields. Moreover, the solid phase approach does not allow an easy modulation of the lipophilic moiety. Indeed, this strategy requires a strong acid final cleavage and deprotection step that is usually not compatible with the stability of unsaturated fatty acid or cholesterol derivatives.

To overcome the above-mentioned problems, we recently described a high-yielding hydrazone-based ligation method, in which the fatty acyl moiety was site-specifically anchored through the formation of a hydrazone bond to the pre-purified hydrazino acetyl peptides using very mild experimental conditions compatible with sensitive fatty acids.[11] The same approach was adapted to the simultaneous lipidation of a peptide mixture, using stoechiometric conditions compatible with diverse peptide or lipid structures, to suppress the need for a resolutive purification after introduction of the lipid tail (Scheme 1). This chemistry was previously applied to the synthesis of a mixture of lipopeptides by reacting palmitic acid α-oxo aldehyde with a mixture of α-hydrazinoacetyl peptides with the aim of producing a synthetic peptide vaccine.[12]

Here, we demonstrated the potential and applicability of stoechiometric hydrazone ligation in parallel synthesis by performing preparation of a library of lipopeptides with good

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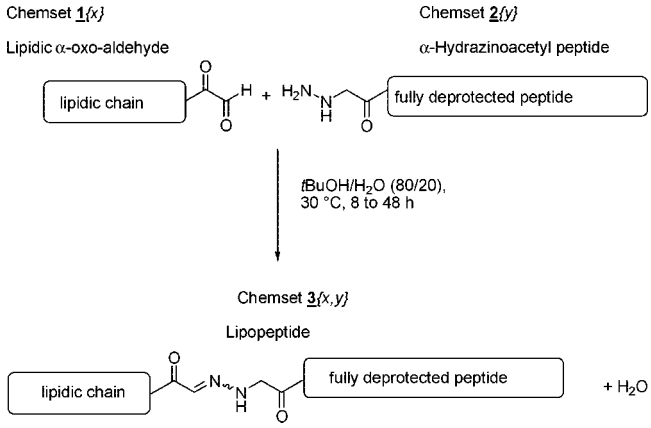
Scheme 1. General Strategy of Lipopeptide Synthesis by Hydrazone Ligation



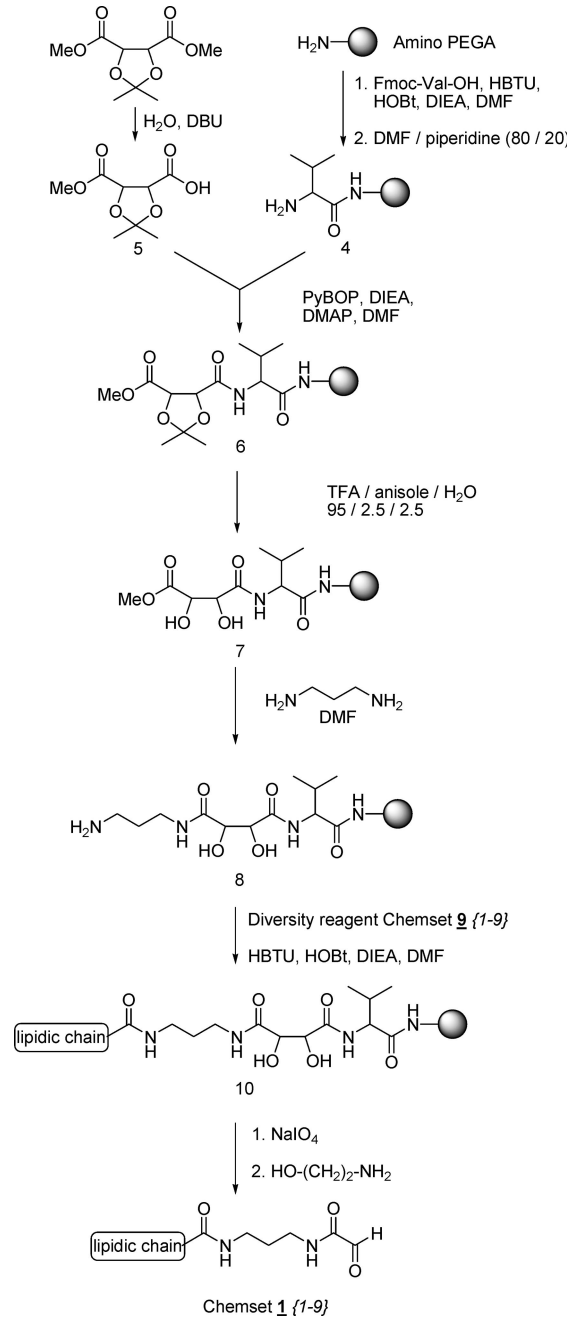
purity. Indeed, this versatile method does not require any activating agent, limiting side-product formation. Ligation was preformed in solution in deep-well plates using 10 lipidic-α-oxo-aldehydes (Chemset **1**) and 11 α-hydrazinoacetylpeptides (Chemset **2**). One-hundred-two lipopeptides (Chemset **3**, 0.54 μmol each) were successfully obtained.

Conception and Synthesis of Ligation Partners and Lipopeptides Library. The lipidic α-oxo-aldehydes (Chemset **1**{*1–10*}), composed of a lipoyl and glyoxylyl group linked by an 1,3-diaminopropane moiety, were synthesized on amino PEGA resin using a (+)-D-tartrate linker (8, Scheme 2), which was prepared by deprotecting on the solid phase the dimethyl-2,3-*O*-D-tartrate moiety of solid-support 6. In the previously described procedure,[13] tartrate linker was cleaved from the resin with TFA after solid-phase introduction of the saturated lipidic chain, just before a final periodic oxidation step allowing formation of the α-oxoaldehyde moiety. However, the use of unsaturated fatty acid is incompatible with strong acid such as TFA. We thus decided to perform the TFA treatment that unmasks the diol before introduction of the lipidic chain. Briefly, (+)-dimethyl-2,3-*O*-isopropylidene-D-tatrate was partially saponified with water and DBU, and anchored on H-Val-NH-PEGA resin under PyBOP activation. Next, 1,3-diaminopropane displaced the second ester moiety and the terminal amino group was readily coupled separately under HBTU/DIEA activation with eight different fatty acids or cholesterol derivatives (Diversity reagent Chemset **9**{*1–9*}) summarized in Table 1. Solid-phase periodic oxidation allowed both the formation of α-oxo-aldehyde moiety and separation of product from the solid support, by several washings steps at 50 °C, to give Chemset **1**{*1–9*}. The great interest of this strategy is that the diol moiety of tartrate linker is unmasked before introducing the lipidic chain, thus avoiding the exposure of the sensitive fatty acids to strong acidic media such as TFA.

This method is convenient in a combinatorial approach. It allowed the rapid synthesis of numerous α-oxo-aldehydes with moderate yields (20 to 42%) but with high purity (about 100%). Lipidic α-oxo-aldehydes were analyzed by LC/MS and characterized by NMR.

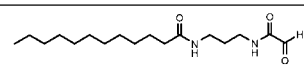
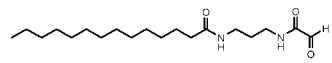
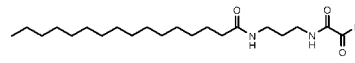
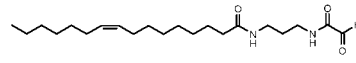
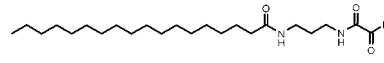
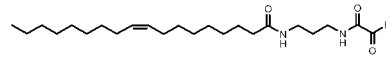
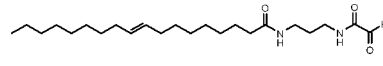
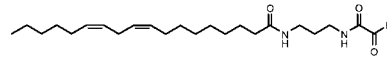
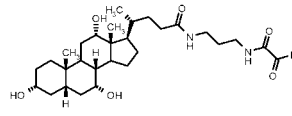
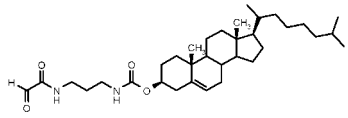
The 11 α-hydrazinoacetyl peptides (Chemset **2**{*1–11*}) were prepared by semiparallel Fmoc-SPPS on SynPhase Rink Amide lanterns (Scheme 3). Scheme 4 Color cogs and

Scheme 2. Solid-Phase Synthesis of Lipidic α-Oxo-aldehydes Chemset **1**{*1–9*}



spindle tagging allowed us to pool lanterns, whereas common washing or deprotection steps were required.[14] Peptide sequences were chosen from patented or published α-MSH analogues. Melitane[15] (Ac-Nle-Ala-His-DPhe-Arg-Trp-NH_2) was described as an α-MSH agonist and compound 153N-6[16] (H-Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro-Val-NH_2) as an antagonist. The hydrazinoacetyl group was introduced as described,[17,18] either on the N-terminal position or on a side chain to provide two different ways of anchoring the lipophilic moiety. Briefly, *N*,*N*′,*N*″-tri(*tert*-butyloxyxarbonyl)-hydrazino acetic acid ($(Boc)_2N$-N(Boc)-CH_2-COOH) was coupled either with the N-terminal amino group (Path 1) or the side chain amino group of Orn or Lys residues (Path 2). In the last case, selective unmasking of the lateral amine during solid-phase synthesis of peptide required the use of

Table 1. Diversity Reagent **9**{*1–9*} and Chemset **1**{*1–10*}

Entry	Diversity Reagent **9**	Chemset **1** Structure	Yield (%)	TLC [a] (Rf)	MS $[M+H]^+$
1	lauric acid		20	0.46	313.3
2	myristic acid		24	0.46	341.4
3	palmitic acid		25	0.49	369.2
4	palmitoleic acid		22	0.49	367.4
5	stearic acid		32	0.49	397.3
6	oleic acid		36	0.49	395.4
7	elaidic acid		34	0.49	395.4
8	linoleic acid		31	0.50	393.4
9	cholic acid		42	0.19	521.2
10	-		-	0.20	543.4

[a] Eluant: DCM/ethyl acetate/EtOH 6.6/2.4/1.

an orthogonal protection. 4-Methyltrityl (Mtt) protective group was chosen for its potential to be selectively cleaved by mild acidic conditions such as TFA/DCM (1/99 v/v)[19] without premature cleavage of the Rink Amide linker. After TFA cleavage, Chemset **2** was characterized by LC/MS. As shown in Table 2, the overall yields were around 50% and peptide purity was around 80%, without requirement of extra purification steps after cleavage from the resin.

Ligation

Chemoselective ligation between each component of the lipidic α-oxo-aldehyde Chemset **1**{*1–10*} with each α-hydrazinoacetyl peptide of Chemset **2**{*1–11*} to yield Chemset **3**{*1–10,1–11*} was performed in solution in *tert*-butanol/water (80/20) at 30 °C in a quasi-stoichiometric ratio, using 1.2 equiv of α-oxo-aldehyde at 1.3 mM concentration (0.65 μmol) (Scheme 1). Parallel synthesis was carried out for 8–48 hours in deep-well plates with orbital stirring in an oven at 30 °C. It is worth noting that only 102 compounds over the 110 theoretical combinations were synthesized, mainly because ofrather low yields of some α-hydrazinoacetyl peptides like **2**{*9*}.

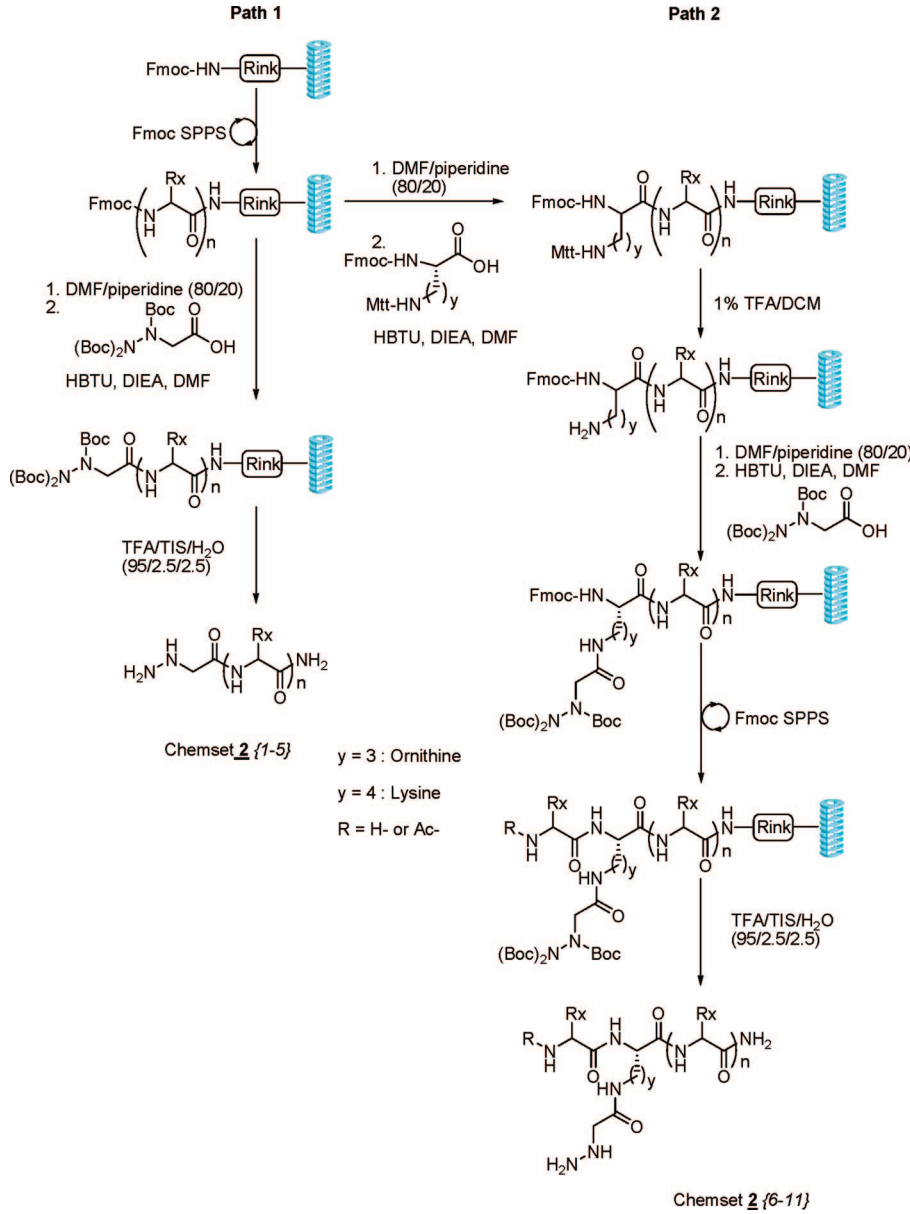
Chemset **3** was characterized by HPLC and LC/MS (Table 3). In some cases, a byproduct corresponding to a double incorporation of the lipidic α-oxo-aldehyde was detected. The presence of the 1,3,4-oxadiazolidine side product was already observed in hydrazone ligation reaction[20] and could be explained by dimerization of α-oxo aldehydes, favored by hydrophobic interactions between lipidic chains, and subsequent reaction of this dimer with hydrazinoacetyl moiety. The occurrence of this side reaction depended on the nature of both the lipidic α-oxo-aldehyde and the α-hydrazinoacetyl peptide.

This versatile and efficient synthesis allowed the preparation of 102 lipopeptides by parallel ligation between 10 lipidic α-oxo-aldehydes and 11 α-hydrazinoacetyl peptides.

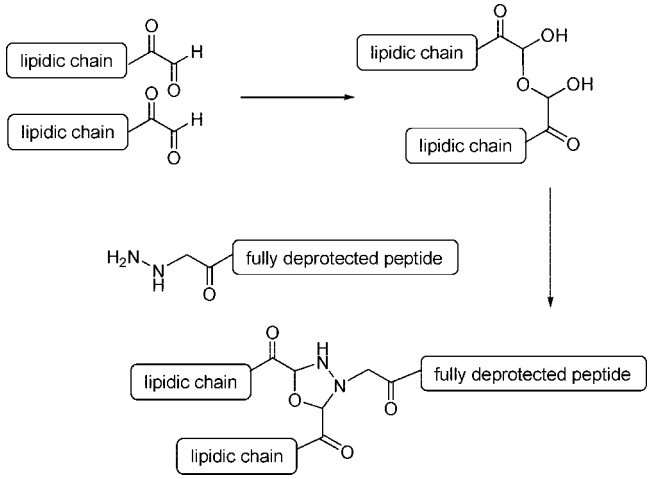
Biological Assays

Chemset **3** was pharmacologically evaluated without purification at 1×10^{-7} M using the M4Be melanoma cell line. To identify potent agonists, we measured second messenger AMPc production and compared it to AMPc production induced by reference agonist Melitane at the same

Scheme 3. Synthesis of α-Hydrazinoacetyl Peptides Chemset **2**{*1–11*} on SynPhase Lanterns



Scheme 4. Putative Side Reaction Occurring during Ligation



concentration. For compounds of Chemset **3** exhibiting a weak induction of AMPc, a competitive test was carried out to identify putative antagonists in presence of 5×10^{-8} M α-MSH. It is worth noting that before the biological evaluation of the library, assays were performed showing that α-oxo-aldehydes of Chemset **1** neither induced not inhibited AMPc production at 1×10^{-7} M. Results of Chemset **3** are presented in Table 3.

Three preliminary remarks can be advanced. First of all, the screening allowed us to identify 99 MC1-R agonists; most of them are as potent or more potent than reference agonist Melitane. Secondly, no antagonist was detected. Surprisingly, modification of the MC1-R antagonist reference compound 153N-6 by introduction of a lipophilic moiety (compounds **3**{*x,8–11*}) led to an inversion of the biological activity (from antagonist to agonist). Finally, each mixture of product and byproduct exhibited a good agonist property. AMPc induction percentages great than 100% were observed in several pure products and in most of the wells where a lipidic byproduct was detected. Thus, although the relative contribution of lipidic byproduct was difficult to evaluate, it was

Table 2. Chemset **2**{*1–11*}

entry	structure	yield[a] (%)+	purity[b] (%)	MS $[M + H]^{-1}$
1	H_2N-Gly-His-DPhe-Arg-Trp-NH_2	50	81	716.3
2	H_2N-Gly-Ala-His-DPhe-Arg-Trp-NH_2	59	90	787.5
3	H_2N-Gly-Nle-Ala-His-DPhe-Arg-Trp-NH_2	56	93	900.6
4	H_2N-Gly-Nle-Ala-Lys-DPhe-Arg-Trp-NH_2	54	82	891.6
5	H_2N-Gly-Nle-Ala-His-DPhe-Lys-Trp-NH_2	49	83	872.6
6	Ac-Nle-Ala-Lys($COCH_2NHNH_2$)-DPhe-Arg-Trp-NH_2	46	81	933.6
7	Ac-Nle-Ala-His-DPhe-Lys($COCH_2NHNH_2$)-Trp-NH_2	47	85	914.5
8	H-Met-Pro-DPhe-Arg-DTrp-Phe-Lys($COCH_2NHNH_2$)-Pro-Val-NH_2	46	88	1278.8
9	H-Met-Pro-DPhe-Arg-DTrp-Phe-Orn($COCH_2NHNH_2$)-Pro-Val-NH_2	8	98	1264.8
10	H-Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro-Val-Lys($COCH_2NHNH_2$)-NH_2	52	97	1406.9
11	H-Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro-Val-Orn($COCH_2NHNH_2$)-NH_2	22	94	1392.7

[a] Calculated on the basis of initial loading of Lanterns (13 μmol) determined by Fmoc titration. [b] Calculated on the basis of HPLC analysis using relative peak areas at 214 nm.

likely the introduction of a second lipophilic chain did not induce a sufficient activity modification to be noticed.

In a general manner, adding lipidic chain at the N-terminus of several Melitane analogues (compound **3**{*x,1–5*}) yielded strong MC1-R agonists (with activation percentage greater than 100%). Introduction of lipidic moiety on the side chain of lysine residue substituting the original Arginine of Melitane also yielded good agonists (Chemset **3**{*x,7*}). This result seemed to prove that a hydrophobic chain could replace the guanidine side chain of arginine without loss of α-MSH activity. On the contrary, when a hydrophobic moiety was introduced on the side chain of lysine (which replaced the histidine residue of the Melitane sequence) (Chemset **3**{*x,6*}), AMPc induction was very variable. Indeed, unsaturated C_{12}, C_{14}, and C_{18} fatty acid or cholesterol derivatives (**3**{*1–2,6*} and **3**{*7–10,6*}) induced low or no AMPc production but ligation with C_{16} or C_{18} (saturated or unsaturated cis) led to good agonist **3**{*3–6,6*}. The length of the hydrocarbon chain as well as the nature and the number of unsaturations seemed to influence the agonistic activity of this peptide. Moreover, these results confirmed the importance of imidazole group in Histidine side chain of α-MSH agonist, which seemed to play an important role in ligand–receptor interaction.

Surprisingly, lipopeptides derived from the α-MSH antagonist 153N-6 were found to be agonist or inactive. Compounds with a hydrophobic group on the side chain of the lysine exhibited good agonist activity (products **3**{*1–10,8*}). Replacement of this lysine by an ornithine led to less-active compounds (**3**{*1,9*}, **3**{*3,9*}, **3**{*6,9*}, and **3**{*9,9*}). Similarly, lipidation of the side chain of lysine or ornithine added on the C-terminus gave agonist compounds that were less active than Melitane (compounds **3**{*1–10,10*} and **3**{*1–10,11*}).

To conclude, this library allowed us to obtain 84 MC1-R agonists exhibiting activity higher than Melitane. Among all performed modifications, we can notice that replacement of histidine of the Melitane sequence by a lysine and introduction of lipophilic moieties on its side chain could led to less-active compounds than the naked peptide. Moreover, each modification performed on 153N-6 yielded to inversion of the biological activity.

Experimental Section

Materials. All solvents of analytical grade were obtained from Carlo Erba (Val de Rueil, France) or Riedel de Haën (Seeze, Germany) and used without purification. D-sized polyamide Fmoc Rink Amide Lanterns with a theoretical 21 μmol loading, cogs, and spindles were purchased from Mimotopes (Clayton, Australia). Amino PEGA resin, 3-(4-(hydrazinosulfonyl-1)phenyl)propionyl AM resin, PyBOP, HOBt, Fmoc-Lys(Mtt)-OH, and Fmoc-Orn(Mtt)-OH were obtained from Novabiochem (Laüfelfingen, Switzerland). All other Fmoc amino acids and HBTU reagent were purchased from Senn Chemicals (Gentilly, France). *N*′,*N*′-Tri(*tert*-butyloxyxarbonyl)-hydrazino acetic acid was a kind gift from UMR CNRS 8525 but is commercially available at Novabiochem (Laüfelfingen, Switzerland). Fatty acids were obtained from Avocado (La Tour du Pin, France) and 1,3-diaminopropane from Acros (Noisy-le-Grand, France). Anisole, DBU, DIEA, DMAP, (+)-dimethyl-2,3-*O*-isopropylidene-D-tatrate, ethanolamine, and sodium periodate were purchased from Aldrich (Saint Quentin Fallavier, France).

The following abbreviations are used: BHT, 2,6-di-*tert*-butyl-4-methylphenol; DBU, 1,8-diazabicyclo(5.4.0)undec-7-ene; DCM, dichoromethane; DIEA, *N*,*N*,*N*-diisopropylethyl amine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; HBTU, 2- (1 *H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TIS, triisopropylsilane, and TNBS, 2,4,6-trinitrobenzenesulfonic acid. Other abbreviations used are those recommended by the UPAC-UB Commission (*Eur. J. Biochem.* **1984**, 1389-37).

RP-HPLC and LC/MS Analyses. RP-HPLC monitoring of Chemset **3** syntheses was performed on a Shimadzu system equipped with a C3 Zorbax 300 Å, 200 × 4.6 mm reversed-phase column heated in an oven at 60 °C. Detection was performed at 215 nm on a Shimadzu SPD10A detection system.

LC/MS analyses of Chemsets **1**, **2**, and **3** were performed on a Waters Alliance 2690 HPLC equipped with a Waters 996 photodiode array and coupled to a Micromass Platform II mass spectrometer (positive electrospray ionization mode, ESI+). For Chemset **1** and **3**, LC/MS analyses were carried out using a C1, Kromasil 5-μm, 50 × 4.6 mm reversed-phase column. For Chemset **2**, a C18 Merck Chromolith Speed Rod 50 × 4.6 mm reversed-phase column was used. Positive ion electrospray mass spectra were acquired at a flow rate of 100 μL min^{-1}. Nitrogen was used both for the nebulizing gas and the drying gas. Data were acquired in

Table 3. Biological Activity and LC/MS Analysis of Chemset **3**{*1–10,1–11*} Compounds

x≤40 40<x≤60 60<x≤80 80<x≤100 x>100

Compound	Purity (%)[a]	MW calc.	m/z [M+H]$^+$	By product %	By product Cltd mass	By product m/z [M+H]$^+$	AMPc Induction[b]
3 *{1,1}*	74	1009.6	1010.3	8	1322.2	1322.7	
3 *{2,1}*	72	1037.7	1038.3	10	1378.3	1379.2	
3 *{3,1}*	48	1065.7	1066.4	39	1431.1	1431.9	
3 *{4,1}*	61	1063.7	1064.3	13	1430.4	1430.9	
3 *{5,1}*	81	1093.7	1094.4	-	-	-	
3 *{6,1}*	77	1091.7	1092.4	-	-	-	
3 *{7,1}*	75	1091.7	1092.4	-	-	-	
3 *{8,1}*	79	1089.7	1090.4	-	-	-	
3 *{9,1}*	83	1217.7	1218.5	-	-	-	
3*{10,1}*	69	1239.8	1240.6	-	-	-	
3 *{1,2}*	70	1808.6	1081.3	-	-	-	
3 *{2,2}*	74	1108.7	1109.4	12	1449.0	1449.9	
3*{3,2}*	64	1136.7	1137.4	20	1505.5	1506.0	
3 *{4,2}*	54	1134.7	1135.4	26	1501.5	1502.0	
3 *{5,2}*	92	1164.8	1165.5	-	-	-	
3 *{6,2}*	76	1162.7	1163.5	-	-	-	
3 *{7,2}*	80	1162.7	1163.5	10	1557.9	1559.1	
3 *{8,2}*	73	1160.7	1161.5	9	1553.9	1555.1	
3 *{9,2}*	78	1288.7	1289.6	-	-	-	
3 *{2,3}*	81	1221.8	1222.6	-	-	-	
3 *{3,3}*	67	1249.7	1250.6	17	1618.7	1619.2	
3 *{4,3}*	66	1247.8	1248.6	25	1614.6	1615.1	
3 *{5,3}*	73	1277.8	1278.7	-	-	-	
3 *{6,3}*	74	1275.8	1276.6	16	1671.1	1672.2	
3 *{7,3}*	70	1275.8	1276.6	10	1671.1	1672.2	
3 *{8,3}*	72	1273.8	1274.6	-	-	-	
3 *{9,3}*	71	1401.8	1402.8	-	-	-	
3 *{10,3}*	70	1423.9	1424.8	-	-	-	
3 *{1,4}*	30	1184.6	1185.5	45	1497.5	1498.0	
3 *{2,4}*	71	1212.8	1213.6	8	1553.6	1554.1	
3 *{3,4}*	37	1240.7	1241.6	48	1609.7	1610.2	
3 *{4,4}*	32	1238.8	1239.6	58	1605.7	1606.2	
3 *{5,4}*	72	1268.9	1269.7	-	-	-	
3 *{6,4}*	62	1266.9	1267.7	18	1662.2	1663.3	
3 *{7,4}*	74	1266.9	1267.7	14	1662.0	1663.3	
3 *{8,4}*	63	1264.8	1265.7	27	1657.8	1658.9	
3 *{9,4}*	57	1392.9	1393.8	32	1914.3	19155	
3 *{10,4}*	87		1415.9	-	-	-	

Compound	Purity (%)[a]	MW calc.	m/z [M+H]$^+$	By product %	By product Cltd mass	By product m/z [M+H]$^+$	AMPc Induction[b]
3 *{1,5}*	50	1165.7	1166.5	27	1478.5	1478.9	
3 *{2,5}*	76	1193.7	1194.5	-	-	-	
3 *{3,5}*	65	1221.7	1222.6	21	1590.6	1591.2	
3 *{4,5}*	72	1219.7	1220.6	20	1586.6	1587.1	
3 *{5,5}*	94	1249.9	1250.6	-	-	-	
3 *{6,5}*	89	1247.8	1248.6	-	-	-	
3 *{7,5}*	86	1247.8	1248.6	9	1643.0	1644.2	
3 *{8,5}*	83	1245.7	1246.6	13	1638.8	1640.0	
3 *{9,5}*	72	1373.8	1374.7	6	1895.2	1896.5	
3 *{10,5}*	82	1295.9	1396.8	-	-	-	
3 *{1,6}*	78	1226.8	1227.6	-	-	-	
3 *{2,6}*	82	1254.8	1255.6	-	-	-	
3 *{3,6}*	50	1282.8	1283.7	29	1651.6	1652.2	
3 *{4,6}*	84	1280.8	1281.7	-	-	-	
3 *{5,6}*	76	1310.9	1311.7	-	-	-	
3 *{6,6}*	76	1308.9	1309.7	-	-	-	
3 *{7,6}*	71	1308.5	1309.7	-	-	-	
3 *{8,6}*[c]	77	1306.8	1307.7	-	-	-	
3 *{9,6}*[c]	83	1434.9	1435.8	-	-	-	
3 *{10,6}*[c]	78	1457.0	1457.9	-	-	-	
3 *{1,7}*	59	1207.7	1208.5	21	1520.2	1521.0	
3 *{2,7}*	67	1235.8	1236.6	15	1575.0	1576.1	
3 *{3,7}*	27	1263.8	1264.6	58	1632.6	1633.2	
3 *{4,7}*	43	1261.8	1262.6	39	1632.6	1633.2	
3 *{5,7}*	86	1291.8	1292.7	-	-	-	
3 *{6,7}*	82	1288.8	1290.7	-	-	-	
3 *{7,7}*	60	1289.8	1290.7	9	1685.8	1686.3	
3 *{8,7}*	83	1287.8	1288.7	-	-	-	
3 *{9,7}*	79	1415.8	1416.8	-	-	-	
3 *{10,7}*	70	1437.9	1438.9	-	-	-	
3 *{1,8}*	92	1571.9	1573.0	-	-		
3 *{2,8}*	84	1600.0	1601.1	-	-	-	
3 *{3,8}*	64	1628.0	1629.1	9	1997.1	1997.7	
3 *{4,8}*	81	1626.0	1627.1	12	1993.0	1993.7	
3 *{5,8}*	63	1656.1	1657.2	-	-	-	
3 *{6,8}*	74	1654.0	1655.2	-	-	-	
3 *{7,8}*	68	1654.0	1655.2	-	-	-	
3 *{8,8}*	75	1652.0	1653.2	-	-	-	
3 *{9,8}*	84	1780.1	1781.3	-	-	-	
3 *{10,8}*	81	1802.2	1803.4	-	-	-	

Table 3. Continued

Compound	Purity (%)[a]	MW calc.	m/z [M+H]$^+$	By product %	By product Cltd mass	By product m/z [M+H]$^+$	AMPc Induction[b]
3 *{1,9}*	76	1558.1	1559.0	-	-	-	ND[d]
3 *{3,9}*	77	1614.2	1615.1	-	-	-	
3 *{6,9}*[c]	96	1640.0	1641.2	-	-	-	
3 *{9,9}*[c]	69	1766.2	1767.3	-	-	-	
3 *{1,10}*	79	1700.0	1701.2	-	-	-	
3 *{2,10}*	73	1728.1	1729.3	-	-	-	
3 *{3,10}*	71	1756.1	1757.3	6	2125.2	2125.9	
3 *{4,10}*	84	1754.1	1755.3	-	-	-	
3 *{5,10}*	86	1784.4	1785.4	-	-	-	
3 *{6,10}*	75	1782.1	1783.4	-	-	-	
3 *{7,10}*	70	1785.2	1783.4	-	-	-	
3 *{8,10}*	74	1780.1	1781.3	-	-	-	
3 *{9,10}*	84	1908.2	1909.5	-	-	-	
3 *{10,10}*	77	1930.3	1931.6	-	-	-	
3 *{1,11}*[c]	81	1686.0	1687.2	-	-	-	
3 *{2,11}*	81	1714.0	1715.2	-	-	-	
3 *{3,11}*	77	1742.1	1743.3	-	-	-	
3 *{4,11}*	87	1740.1	1741.3	-	-	-	
3 *{5,11}*	75	1770.1	1771.3	-	-	-	
3 *{6,11}*	81	1768.1	1769.3	-	-	-	
3 *{7,11}*	74	1768.0	1769.3	-	-	-	
3 *{8,11}*	82	1766.2	1767.3	-	-	-	
3 *{9,11}*	77	1894.1	1895.4	-	-	-	
3 *{10,11}*	81	1916.2	1917.5	-	-	-	

[a] Calculated on the basis of HPLC analysis using relative peak area at 214 nm. [b] AMPc induction, in percentage (x) in comparison with α-MSH maximal response at 100 nM. [c] Percentage of inhibition of α-MSH response at 0.5 nM ≤40% was measured for this compound.

the scan mode from *m/z* 400 to 2500 in 0.1s intervals; 10 scans were summed to produce the final spectrum.

TLC and NMR Analyses. TLC analyses were performed on aluminium sheets (Alugram Sil G/UV$_{254}$ Macherey-Nagel) covered with silica gel, and revelation after elution was carried out with $KMnO_4$ and/or phosphomolybdic acid. Eluant: dichloromethane/ethyl acetate/ethanol 6.6/2.4/1.

^{1}H NMR spectra were recorded on a Brüker spectrometer at 300 MHz in pyridine-d_5 with tetramethylsilane (TMS) as internal standard. ^{13}C NMR spectra were recorded on the same apparatus at 75 MHz.

Synthesis of Lipidic α-Oxo-aldehydes Chemset 1 *{1–9}*. (a) Preparation of H-Val-NH-PEGA Resin 4. Five grams of amino PEGA resin (0.4 mmol/g) was washed with DCM (2 × 2 min), DIEA/DCM (5/95 v/v) (1 × 2 min and 1 × 5 min), DCM (2 × 2 min), and DMF (2 × 2 min); 2.716 g (4 equiv, 8 mmol) of Fmoc-Val-OH, 3.034 g (4 equiv, 8 mmol) of HBTU, 1.181 g (4 equiv, 8 mmol) of HOBt, and 4.185 mL of DIEA (12 equiv, 24mmol) were solubilized in 10 mL of DMF and poured on the resin. After 45 min of being stirred at room temperature, the resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min). Completion of the coupling reaction was checked by a TNBS test.[21] Fmoc cleavage was performed by a DMF/piperidine (80/20 v/v) solution (1 × 5 min and 1 × 15 min) and resin was washed with DMF (4 × 2 min).

(b) Compound 5. DBU (1.195 mL) was added to a solution of (+)-dimethyl-2,3-*O*-isopropylidene-D-tatrate (14.695 mL, 80 mmol, 40 equiv) and water (144 μL, 8 mmol, 4 equiv). Partial saponification was performed for 1 h at room temperature without stirring.

(c) Resin 6. The solution of compound **5** (used without purification), DIEA (2.785 mL, 16 mmol, 8 equiv), DMAP (98 mg, 0.8 mmol, 0.4 equiv), and PyBOP (4.162 g, 8 mmol, 4 equiv) was added to resin **4**. The reaction was stirred for 1 h at room temperature. The resin was washed with DMF (3 × 2 min). Because of a positive TNBS test, the coupling reaction was repeated with a freshly prepared solution of compound **5**.

(d) Resin 7. Starting from resin **6**, cleavage of diol protection was performed with 40 mL of a TFA/anisole/water (95/2.5/2.5 v/v/v) solution for 1 h at room temperature. Washings were performed with DIEA/DCM (5/95 v/v) (2 × 2 min), DCM (2 × 2 min), and DMF (3 × 2 min). Resin **7** was split into 5 parts (400 μmol each) stored in suspension in DCM in a jacketed solid-phase reactors.

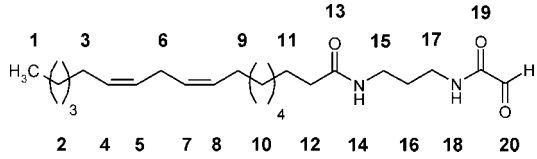
(e) Resin 8. A solution of 2.570 mL of 1,3-diaminopropane in 1.5 mL of DMF was poured on one part of resin **7** preconditioned in DMF (2 × 2 min). The medium was stirred for 20 min at room temperature. Washing with DMF (2 × 2 min) and a TNBS test were performed.

(f) Preparation of Chemset 10{*1–9*}. A solution of 0.360 mmol (0.9 equiv) of one reagent of chemset **9**{*1–9*}, 136.5 mg of HBTU (0.9 equiv, 0.360 mmol), 48.6 mg of HOBt (0.9 equiv, 0.360 mmol), and 188 μL of DIEA (2.7 equiv, 1.080 mmol) in DMF was poured on resin **8**. The reaction was stirred for 1 h at room temperature. Washings were performed with DMF (3 × 2 min), and DCM (3 × 2 min). The coupling reaction was repeated and its achievement was checked by a TNBS test.

Preparation of Compound **10**{*10*} will be described elsewhere.

(g) Preparation of Chemset 1{*1–9*} by Oxidative Cleavage. A solution of sodium periodate (171.1 mg, 0.8 mmol, 2 equiv) in 4 mL of *t*BuOH/acetic acid/water (3/2/1 v/v/v) was poured on the resin **10** conditioned in *t*BuOH/acetic acid/water (3/2/1 v/v/v) (1 × 2 min, 1×35 min). Periodic oxidation was performed for 5 min at room temperature, and

Scheme 5. Example of NMR Analysis for Compound **1**{*8*}



963 μL of ethanolamine (16 mmol, 40 equiv) was added to quench the oxidation. The reaction mixture was stirred for 10 min. The filtrate was collected and resin was washed with *t*BuOH/acetic acid/water (3/2/1 v/v/v) (2×2 min) at room temperature. The jacketed reactor was then thermostated at 50 °C, and washing was carried out with *t*BuOH/MeOH (1/1 v/v) (3×2 min). Filtrate and washing solutions were combined, and 60 mL of an aqueous solution of 0.1 M Na_2SO_3 and 100 mL of DCM were added. The organic phase was then washed with 0.1 M Na_2SO_3 aq (2×50 mL), and the aqueous phase was washed with 150 mL of DCM. Organic phases were combined, dried on $MgSO_4$, filtered, and concentrated in vacuo.

(h) Chemset 1 Analyses. For LC/MS analyses, samples were prepared in *tert*-Butanol/water (50/50 v/v) and a gradient of (0–100)% B over 20 min plus 10 min at 100% B was used. Eluent A, water/0.1% TFA; eluent B, acetonitrile/0.1% TFA.

LC/MS and NMR analyses highlighted the coexistence of α-oxo-aldehyde under hydrate and hemiacetal forms.

TLC analyses were performed with an DCM/AcOEt/EtOH (6.6/2.4/1) eluent, and a single spot was revealed whatever revelation reagent used.

For an example of NMR analysis, see Scheme 5. Attributions were performed thanks to HSQC, TOCSY, and COSY spectra. For the NMR spectra of the entire Chemset **1**, see the Supporting Information. ^{1}H NMR (300 MHz, pyridine-d_5): δ 0.83 (m, 3H, H_1), from 1.22 to 1.34 (m, 14H, $H_{2,10}$), 1.77 (m, 2H, H_{11}), 1.86 (m, 2H, H_{16}), 2.06 (m, 4H, $H_{3,9}$), 2.34 (t, 2H, H_{12}, J = 7.4Hz), 2.90 (t, 2H, H_6, J = 5.6Hz), 3.49 (m, 2H, H_{15}), 3.60 (m, 2H, H_{17}), 5.47 (m, 4H, $H_{4,5,7,8}$), 5.92 (s, 0.57H, H_{20}, hydrate form), from 8.32 to 8.42 (m, 1H, H_{14}), 8.80 (m, 0.43H, H_{18}, aldehyde form), 9.48 (m, 0.57H, H_{18}, hydrate form), 9.59 (s, 0.43H, H_{20}, aldehyde form).

^{13}C NMR (75 MHz, pyridine-d_5): δ 14.4 (C_1), 26.1 (C_6), 26.4 (C_8), 27.6 ($C_{3,9}$), 30.1 ($C_{2,10}$), 30.7 ($C_{11,16}$), 36.9 ($C_{3,5,7}$), 98.3 (C_{20}, hydrate form), 128.5 and 130.5 ($C_{14,15,18,18}$), 189.1 (C_{20}, aldehyde form).

Synthesis of α-Hydrazinoacetyl Peptides Chemset 2{*1–11*}. (a) Standard Fmoc-Deprotection Protocol. Fmoc-deprotection steps were carried out by immersing lanterns in a mixture of DMF and piperidine (80/20 v/v) for 60 min. A flask equipped with a drilled topper was used. The solution was removed simply by reversing the flask.

(b) Standard Washing Protocol. Washing steps after the coupling or deprotection step were performed by dipping lanterns in DMF (3 × 3 min), MeOH (1 × 3 min), and DCM (3 × 3 min) successively. One single flask equipped with a drilled topper was used. Lanterns were allowed to air dry for 5 min after the last DCM washing.

(c) Standard Coupling Protocol. DMF solutions containing each of the Fmoc-protected amino acids, HBTU and DIEA, were freshly prepared before the coupling step ([Fmoc-AA-OH] = [HBTU] = 120 mM, [DIEA] = 240 mM). Color-tagged lanterns were immersed in coupling solution for 2 h at room temperature. Solutions were decanted and lanterns were washed following the standard washing protocol.

(d) Mtt Cleavage Protocol.[19] 4-Methyltrityl group was removed by dipping lanterns for 2 min (several times) in a TFA/DCM (1/99 v/v) solution (18 equiv of TFA). The Mtt carbocation released is quenched by water. Mtt alcohol has a yellow color, thus bleaching of cleavage solution and RP-HPLC analysis allowed to monitor unmasking rate. Every RP-HPLC analyses required a specific sample preparation. Two-hundred microliters of cleavage solution was concentrated under nitrogen circulation in an RP-HPLC vial and diluted in 200 μL of water/THF (50/50 v/v). THF was stabilized by 25–40 ppm of BHT, which was used as internal standard during RP-HPLC monitoring.

(d) Cleavage. A 2 mL portion of TFA/water/triisopropylsilane (95/2.5/2.5 v/v/v) was dispensed into 110 individual polypropylenes of two different 96-well 1.5 mL plates (Micronic system, Lelystad, Holland). Cleavage was performed for 2 h, and the cleavage cocktail was removed directly from plates using a Jouan RC1010 vacuum centrifuge. Compounds were precipitated with diethylether, centrifuged, and decanted one by one. Precipitation, centrifugation, and decantation operations were repeated twice. A 100 μL portion of an acetonitrile/water (50/50 v/v) mixture containing 0.1% TFA was dispensed into each tube to dissolve samples. The samples were then frozen at −80 °C and lyophilized.

(e) LC/MS Analyses. The samples were prepared in a mixture of water/acetonitrile (50/50 v/v). A flow rate of 5 mL min^{-1} and a gradient of (0–100)% B over 3 min were used. Eluent A, water/0.1% TFA; eluent B, acetonitrile/0.1% TFA. Purity estimations were determined on the basis of the area percentages of the peaks detected at 215 nm.

Parallel Synthesis of Lipopeptides Chemset 3 by Chemical Ligation. The parallel ligation was performed in 96-well 2 mL plates in an oven at 30 °C with orbital stirring for 8–48 hours in 500 μL of *tert*-Butanol/water (80/20 v/v).

In each well, 250 μL of a solution of α-oxo-aldehyde (2.6 mmol/L, 0.65 μmol, 1.2 equiv) and 250 μL of a solution of α-hyrazinoacetyl peptide (1.08 mmol/L, 0.54 μmol, 1 equiv) were distributed.

The reaction progress was monitored by RP-HPLC. Samples were taken in some wells at the initial time and after 8, 24, and eventually 48 h. Samples were prepared by dilution of 20 μL of reaction solution in 70 μL of isopropanol and eventually frozen to wait for HPLC analysis.

After completion of the reaction, a 500 μL portion of water was dispensed into each well. Samples were frozen and lyophilized.

(a) RP-HPLC and LC/MS Analyses. RP-HPLC were carried out using a flow rate of 1 mL min^{-1} and gradient of (0–100)% B over 20 min plus 10 min at 100% B. Eluent A, water/0.5% TFA; eluent B, water/isopropanol (60/40)/0.5 % TFA.

Characterization of Chemset **3** was performed by LC/MS. Samples were prepared in a mixture of acetonitrile/water (50/50 v/v containing 0.5% TFA) and a flow rate of 1 mL min^{-1} and a gradient of (0–100)% B over 20 min plus 10 min at 100% B were used. Eluent A, water/0.1% TFA; eluent B, acetonitrile/0.1% TFA. Purity estimations were determined on the basis of area percentages of the peaks detected at 215 nm.

Biological Evaluation

Cell Line. The human M4Be cell line melanocyte cell line able to produce melanins was used in this study. The cells were maintained in Dulbecco's Modified Eagle Medium with 10% FCS, 1 mM glutamine, 100 U mL^{-1} penicillin and 1 $\times 10^{-4}$ g mL^{-1} streptomycin. All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere, and cell culture media were renewed every 2 days. Cells were plated in a 96-well plate (Nunc, Roskilde, Denmark) 24 h before the peptide contact.

cAMP Measurement. Briefly, cells plated the previous day were incubated for 1 h with 1×10^{-4} M adenine (Sigma, Saint Quentin Fallavier, France) and then for 10 min in Krebs Ringer Hepes medium with 1×10^{-4} M isobutyl-1-methylxanthine (Sigma), 1×10^{-4} M 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Calbiochem, Fontenay sous Bois, France), and library compounds at 1×10^{-7} M concentration. (For antagonist activity determination, α-MSH was added to the buffer for a final concentration of 5×10^{-8} M α-MSH). After this time, cells lysis was made according to manufacturer protocol. cAMP content was measured using a competitive binding assay kit (RPN225, Amersham Pharmacia Biotech) according to the manufacturer instructions. Each experiment was performed a least twice in triplicate.

Results are expressed by comparison with AMPc production inducted by 0.1 μM Melitane, one of the most potent α-MSH agonists[15] (EC_{50}= 1.80 nM).

Supporting Information Available. ^{1}H-NMR spectra of Chemset **1** (PDF). This information is available free of charge via the Internet at http: //pubs.acs.org.

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