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A novel aryl-hydrazide from the marine lichen *Lichina pygmaea*: Isolation, synthesis of derivatives, and cytotoxicity assays

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ABSTRACT

A new aryl-hydrazide L-glutamic acid derivative, pygmeine (**3**), was isolated from a methanolic extract of *Lichina pygmaea*, a marine lichen. Synthetic derivatives obtained via a two-step coupling of L-glutamic acid with phenylhydrazine moieties were useful to elucidate the structure of **3** and to carry out biological assays. Thus, the cytotoxicity of the *ortho*-, *meta*-, and *para*-hydroxyl isomers along with their respective benzyl intermediates, and a natural methoxylated analog, were evaluated on murine and human melanoma cells (B16, A375). The *para*-hydroxyl isomer **6** was found to be the most active (IC_{50} = 1.6 μM) on B16 cells.

As a continuation of our ongoing work[1] on bioactive secondary metabolites isolated from lichens, we focused on *Lichina pygmaea*, which is a shrubby black marine lichen, found on the rocky shore at the seaside, in the intertidal region. This lichen results from the symbiosis of a fungus and a *Calothrix* cyanobacterium. Forming dark cushions of some millimeters thick, it suffers from harmful UV radiation, exposure to sun and reverberation, a salty environment, immersion phases, and the violence of waves. So, it is assumed that this lichen may possess protective secondary metabolites, and antioxidant properties are claimed for a mycosporine compound.[2,3] Two amino acid derivatives had previously been isolated, namely mycosporine-serinol (**1**), which has a good UVB filter profile, and the L-glutamic acid 5-[(2,4-dimethoxyphenyl)-hydrazide] (**2**).[4] This last compound was found to have antioxidant activities,[5] and is claimed to be a myeloperoxidase inhibitor as isolated from *Penicillium* cultures.[6] A third compound **(3)** has additionally been isolated from a crude methanolic extract of *L. pygmaea*, by Centrifugal Partition Chromatography (CPC) (Fig. 1). Low quantities obtained and ambiguous spectroscopic data for structure identification led us to synthesize analogs which were also tested for cytotoxicity in a preliminary assay on melanoma cell lines.

Extraction by three successive solvents was performed on 270 g of the dried lichen,[7] namely ethyl acetate (1.5 L × 3) at room temperature for 2 h, then methanol (1.5 L × 7) at 45 °C for 2 h, and methanol 50% in water (1.5 L × 3) at 45 °C for 2 h. A first fractionation was conducted on the filtrate of the methanolic extract of *L. pygmaea* by a multiple dual-mode centrifugal partition chromatography method (MDM CPC) previously described,[4] using BuOH/AcOH/H_2O (4:1:5) as the biphasic solvent system. Fraction Lp6 resulted in the isolation of compound **2** (121 mg) and fraction Lp8 in the isolation of compound **1** (25 mg). Fraction Lp7 was evaporated to dryness (1.065 g), and the residue was further partitioned between CH_2Cl_2 and H_2O. The aqueous layer was concentrated and subjected to chromatography over a reversed-phase silica gel

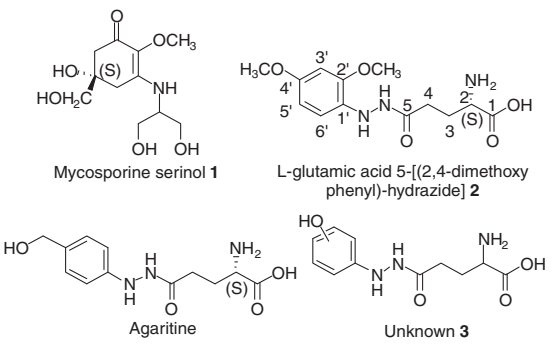

Figure 1. Structures of compounds isolated from *L. pygmaea*, and the similar agaritine.

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Table 1
NMR spectroscopic data (270 MHz, D_2O) of isolated compounds **2** and **3**, compared with synthetic *o*-, *m*- and *p*-isomers **4**–**6**

Position	2		3		4		5		6	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)
1	176.3		174.4*		174.4*		175.2*		175.7*	
2	56.6	3.70, m	54.7	3.77, t (6.1)	54.7	3.77, t (6.2)	55.6	3.78, t (6.2)	56.0	3.77, t (6.2)
3	28.7	2.08, m	26.7	2.18, m	26.7	2.17, m	27.6	2.17, m	28.0	2.17, m
4	32.1	2.41, m	30.1	2.50, m	30.1	2.50, m	30.9	2.50, m	31.4	2.49, m
5	176.9		175.2*		175.2*		176.1*		176.4*	
1′	132.8		136.1		136.1		150.5		142.4	
2′	157.0		144.4		144.4		101.7	6.36, d (2.2)	117.4*	6.81, s
3′	102.1	6.55, d (2.5)	116.0	6.85, s	116.0	6.86, s	158.1		118.0*	6.81, s
4′	151.2		121.6	6.85, s	121.6	6.86, s	109.5	6.44, m	151.9	
5′	107.6	6.43, dd (8.7, 2.5)	122.3	6.85, s	122.3	6.86, s	132.2	7.15, t (8.1)	118.0*	6.81, s
6′	116.8	6.71, d (8.7)	114.4	6.85, s	114.4	6.86, s	107.1	6.44, m	117.4*	6.81, s
OCH_3 (2′)	58.4	3.68, s								
OCH_3 (4′)	58.5	3.76, s								

[a] δ_C described by Hilbig et al.[9]
* Not attributed signals.

(C-18 Hydro Chromabond®) using H_2O/AcOH (0.1%) and increasing gradients of MeOH (10%, 50% to 100% MeOH). The first fraction that eluted with H_2O/AcOH (0.1%) was collected in eight subfractions. The seventh subfraction was evaporated to dryness (40 mg) and the residue was made to precipitate in ACN/MeOH (90:10) to obtain 20 mg of a yellow powder. It was finally subjected to a semi-preparative reversed-phase HPLC (Hypersil® BDS, 250 × 10 mm, 5 µm, 1.0 mL/min; UV detection at 254 nm) using H_2O (0.1% AcOH) for 45 min, and a MeOH/H_2O non-linear gradient (10% for 17 min, 50% for 22 min, and then 100% for 15 min), yielding compound **3**, t_R 51.2 min (3 mg).

A first attempt for structure determination of this newly described compound **3** was performed using NMR and MS.[8] The HRESIMS analysis of **3** displayed a molecular ion peak at *m/z* 254.1144 $[M+H]^+$, confirmed by the presence of two other peaks at *m/z* 276.0966 and *m/z* 292.0693 standing for $[M+Na]^+$ and $[M+K]^+$, respectively, suggesting the molecular formula $C_{11}H_{15}N_3O_4$. ^1H NMR spectrum showed two signals at 2.18 and 2.50 ppm, respectively, each integrating for two protons and another signal for one proton at 3.77 ppm, indicating the neighboring of an oxygen or a nitrogen atom. Four protons were also observed in the aromatic zone, as a broad singlet at 6.85 ppm. Close structural relationships between compound **3** and the previously isolated compound **2** appeared, as a similar aliphatic proton pattern was observed in ^1H NMR (Table 1). Both compounds also presented a yellow coloration with sulfuric anisaldehyde and a positive pink reaction to ninhydrin reagent on TLC plates, confirming the presence of an amino residue in the molecule. These data along with distinct R_f values (0.45 for **2** and 0.26 for **3** in $CHCl_3$/MeOH/H_2O 6:4:1), suggested a similar skeleton differing from the aromatic ring substitution. Therefore, three possible structures **4–6** have emerged as an option and the hydroxyl group position had to be ascertained (Fig. 2). The *para*-hydroxyl compound **6** was the only one described in literature, as xanthodermine, isolated from the Basidiomycete mushroom *Agaricus xanthoderma*.[9] NMR proton profiles were similar, but not conclusive and ^{13}C NMR data suggested the presence of six non equivalent aromatic carbons, which was not consistent with the *para*-hydroxyl compound. Due to the

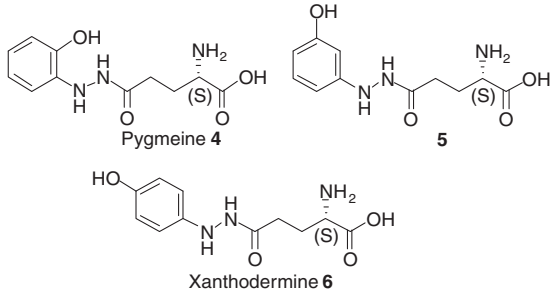
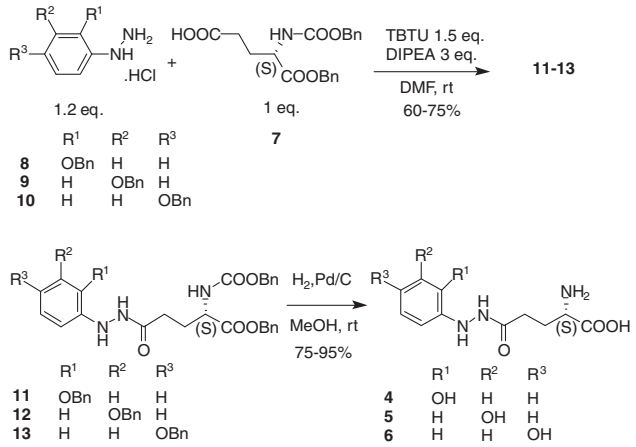
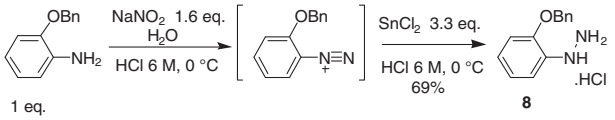


Figure 2. Possible structures of compound **3**.



Scheme 1. Synthesis of compounds **4**–**6**.



Scheme 2. Preparation of (2-benzyloxy-phenyl)-hydrazine, hydrochloride **8**.

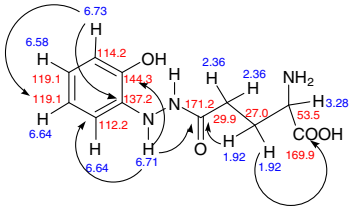


Figure 3. Structure of pygmeine and its ^1H and ^{13}C NMR chemical shift assignments in DMSO-d_6, with selected HMBC correlations.

Table 2
Cytotoxicity results on melanoma cells (A375 and B16) after a 24 h or a 48 h exposure

Compound	A375				B16			
	24 h		48 h		24 h		48 h	
	IC_{50} (μM)[a]	% Inh at 50 μM	IC_{50} (μM)	% Inh at 50 μM	IC_{50} (μM)	% Inh at 50 μM	IC_{50} (μM)	% Inh at 50 μM
1	>50	11	>50	20	>50	34	27 ± 1.5	85
4	>50	11	>50	19	20 ± 2.5	100	14 ± 2	100
5	>50	8	>50	14	12 ± 1	100	12 ± 0.2	100
6	>50	12	>50	31	1.6 ± 0.2	100	0.7 ± 0.15	100
11	29 ± 2	77	30 ± 6	69	21 ± 1	96	15 ± 3	100
12	28 ± 1	80	29 ± 1	73	8 ± 2	100	14.5 ± 2	100
13	>50	4	>50	19	5 ± 1.5	100	0.6 ± 0.1	100
Etoposide	19 ± 5	100	12 ± 6	100	9 ± 4.5	100	0.13 ± 0.07	100

[a] Each result is the mean ± SD of at least two experiments in triplicate.

limited quantities of the purified compound **3**, only the ^{1}H NMR data were unambiguously informative and the synthesis of the three optional derivatives appeared to be appropriate to determine the structure and to facilitate biological assays.

Therefore synthesis of the three isomers was performed through amide coupling of L-glutamic acid (with protecting groups on the amino acid functions) and benzyloxy phenylhydrazines, followed by cleavage of the protecting groups (Scheme 1). Different approaches were given in previous studies for the coupling of L-glutamic acid and a phenylhydrazine. Xanthodermine was synthesized by Hilbig[9] using HOTDO (2,5-diphenyl-4-hydroxy-3-oxo-2,3-dihydrothiophene-1,1-dioxide) as a coupling reagent.[10] Agaritine, a similar structure, was obtained using (1) the amino acid azide derivative,[11,12] (2) a mixed anhydride formed from the amino acid and ethyl chloroformate,[13] or (3) DCC (dicyclohexylcarbodiimide) coupling the *Z*-Glu-OBn and the corresponding phenylhydrazine (in low yields: 1%, 25%, and 33%, respectively).[14]

Each isomer here was promptly synthesized in a two-step-reaction procedure from the commercially available *Z*-Glu-OBn **7** and the corresponding benzyloxyphenylhydrazine **8–10** (Scheme 1). The 2-benzyloxyphenylhydrazine **8** was prepared from the 2-benzyloxyaniline via the formation of a diazonium intermediate (Scheme 2).[15,16] The first step consisted in forming the C–N bond between the protected glutamic acid **7** and the phenylhydrazine derivative **8–10** using TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as coupling reagent in the presence of the *N*,*N*-diisopropylethylamine Hünig's base (DIPEA) in DMF at room temperature.[17] Compounds **4–6** were then obtained after cleavage of the benzyl protecting groups of the isolated 11–**13,** with an overall yield of 48–66%.[18]

The spectroscopic data of each synthetic isomer **4–6** were compared to those of the isolated compound **3** (Table 1). The proton pattern in the aromatic zone was not consistent with the *meta*-hydroxyl derivative **5**, as the four proton signals were split into three peaks, corresponding to H-2′, H-4′/H-6′ and H-5′ chemical shifts. Xanthodermine **6** and compound **4** should typically have the AA′BB′ and the ABCD splitting pattern respectively, but they both exhibited a broad singlet signal in the aromatic zone as well as the isolated compound **3**. A 0.05 ppm shift can be noticed for this ^{1}H NMR signal in D_2O and in DMSO-d_6, the aromatic zone was extended from 6.56 to 6.76 ppm for **3** and **4**, while the aromatic protons of compound **6** appeared between 6.51 and 6.63. The comparison of the ^{13}C NMR spectra clearly suggested the natural compound **3** was corresponding to the synthetic structure **4**, namely the *o*-hydroxyl isomer. Such a similarity was confirmed as the same correlations could be observed through the 2D COSY and HMBC spectra in DMSO-d_6 (Fig. 3), for the semi-purified compound **3** and the synthetic compound **4**. A correlation between the hydrazide proton (δ_H 6.71, s) and carbon signals at δ_C = 112.2, 144.3, and 171.2 ascertained the ortho position of the hydroxyl group. Due to the limited quantities of the isolated compound **3,** the optical rotation could not be recorded but it was demonstrated on a chiral HPLC column that this compound presented exactly the same retention time than the synthetic compound **4**.[19] It could then be assumed that the 2-position stereochemistry may be 'S' as the biosynthetic pathway to this molecule probably implies the natural L-glutamic acid.

This compound, which is reported here for the first time as a natural product, and from a lichen, was named pygmeine. An analogous compound, the 1-(5-glutamyl)-2-methylhydrazine, where the aryl group is replaced by a methyl group, was shown to be a product of sheep brain glutamine synthetase by Meister and co-workers in 1972.[20] They demonstrated that methylhydrazine could act as a substrate of this enzyme, in place of ammonia. It could be assumed that pygmeine may also be synthesized by a glutamine synthetase-like enzyme.

Compounds **2**, **4–6**, and **11–13** were tested for their cytotoxic activity on human (A375) and murine (B16) melanoma cell lines, and compared to the positive control etoposide (Table 2).[21] As shown by the toxicity at the highest 50 μM concentration tested, the A375 cell line was found less sensitive than the B16 to these compounds. The cytotoxicity observed for protected intermediates (**11–13**) was higher than the corresponding final compounds (**4–6**) on A375 but comparable on B16. It could be assumed that the protected compounds are less polar and therefore penetrate cell membranes more readily. Once inside the cells they may be acted upon by non-specific esterases. After a 24 and 48 h-exposure on B16, the most active compound was the natural xanthodermine **6,** which strongly inhibited the cell growth with an IC_{50} at 1.6 μM (9 μM for etoposide). Therefore xanthodermine, which was previously described as being active against Bacillus strains,[9] was found to be tenfold more cytotoxic than compound **4**, suggesting the *p*-hydroxyl position was enhancing the activity.

As a conclusion, a new aryl-hydrazide L-glutamic acid derivative was isolated from a marine lichen *L. pygmaea*, and was shown to have some activity on melanoma cancer cells. Synthesis of analogs gave a hit compound which is xanthodermine, with a promising cytotoxicity on B16. As most melanoma cells are rich in tyrosinase, it can be expected that this kind of compound would be metabolized by this enzyme, like the γ-glutaminyl-*p*-hydroxybenzene (GHB) whose structure is very close, and therefore have specificity against melanoma cells.[22] This is in accordance with recent works on aryl hydrazines and hydrazides, presenting them as promising anticancer compounds.[23]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.013.

References and notes

1. Boustie, J.; Lohézic-Le Dévéhat, F. In *Botanical Medicine in Clinical Practice*; Preedy, V. R., Ed.; CAB International: Wallingford, 2008; pp 356–365.
2. De La Coba Luque, F.; Aguilera Arjona, J.; Lopez Figueroa, F. Spain patent 2007026036, 2007.
3. De la Coba Luque, F.; Aguilera Arjona, J.; Figueroa, F. L.; de Galvez, M. V.; Herrera, E. *J. Appl. Phycol.* **2009**, *21*, 161.
4. Roullier, C.; Chollet-Krugler, M.; Bernard, A.; Boustie, J. *J. Chromatogr., B* **2009**, *877*, 2067.
5. Roullier, C.; Chollet-Krugler, M.; Lohézic-Le Dévéhat, F.; Rouaud, I.; Boustie, J. *Abstract of Papers*, the 50th Annual Meeting of the American Society of Pharmacognosy, Honolulu, HI; American Society of Pharmacognosy: Honolulu, HI, 2009; Abstract P-187.
6. Tanimoto, T.; Tanaka, I.; Nakajima, M. Japan patent JP 2001131133, 2001.
7. Lichen material was collected on the west coast of France, on the rocky shore of Dinard (48°38.09′ N, 02°08.15′ W), in December 2007. It was sorted out, washed and dried under ambient atmosphere. After macroscopic and microscopic observations, comparing to a reference sample of the 'Des Abbayes' herbarium, it was identified as *Lichina pygmaea* (Lightf.) C. Agardh. and a voucher specimen is kept in the laboratory with the reference JB/07/98.
8. *Spectral data for the isolated compound pygmeine* (**3**). ^{1}H NMR (D_2O, 270 MHz) δ 2.18 (2H, m), 2.50 (2H, m), 3.77 (1H, t, J = 6.1 Hz), 6.85 (4H, s); ^{13}C NMR (D_2O, 62.5 MHz) δ 26.7, 30.1, 54.7, 114.4, 116.0, 121.6, 122.3, 136.1, 144.4, 174.4, 175.2; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.91 (2H, m), 2.31 (2H, m), 3.31 (1H, t, J = 6.3 Hz), 6.58 (1H, m), 6.64 (2H, m), 6.71 (1H, s), 6.73 (1H, ps d, J = 7.7 Hz); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 26.9, 29.8, 53.4, 112.1, 114.2, 119.1, 119.1, 137.2, 144.3, 170.1, 171.1; HRESIMS m/z 254.1144 $[M+H]^+$ (calcd for $C_{11}H_{16}N_3O_4$, 254.1141).
9. Hilbig, S.; Andries, T.; Steglich, W.; Anke, T. *Angew. Chem.* **1985**, *97*, 1063.
10. Hollitzer, O.; Seewald, A.; Steglich, W. *Angew. Chem.* **1976**, *88*, 480.
11. Kelly, R. B.; Daniels, E. G.; Hinman, J. W. *J. Chem. Soc.* **1962**, *27*, 3229.
12. Hinman, J. W.; Kelly, R. B. U.S. patent 3,288,848, 1966.
13. Wallcave, L.; Nagel, D. L.; Raha, C. R.; Jae, H.-S.; Bronczyk, S.; Kupper, R.; Toth, B. *J. Org. Chem.* **1979**, *44*, 3752.
14. Datta, S.; Hoesch, L. *Helv. Chim. Acta* **1987**, *70*, 1261.
15. Witte, J.; Boekelheide, V. *J. Org. Chem.* **1972**, *37*, 2849.
16. *(2-Benzyloxy-phenyl)-hydrazine, hydrochloride* (**8**): 2-benzyloxy-aniline (535 mg, 2.6 mmol) was added dropwise to an ice-cooled solution of HCl 6 M (10 mL). The mixture was stirred at 0 °C, and treated dropwise with sodium nitrite (291 mg, 4.2 mmol), dissolved in water (5 mL). The mixture was stirred vigorously during 60 min at 0 °C. Then tin (II) chloride (1.963 g, 8.5 mmol) dissolved in HCl 6 M (15 mL) was added dropwise and the solution was stirred at 0 °C for an additional 2 h. The reaction mixture was alkalized at pH >11 with NaOH (12.5 M), and extracted with diethyl ether (3 × 100 mL). The organic layer was dried on Na_2SO_4 and hydrochloric acid in methanol (2 mL) was added to the solution to provide hydrazine **8** as a white precipitate (450 mg, 69%): mp 136–138 °C; UV (MeOH) λ_{max} (log ε) 208 (4.43), 232 (3.85), 276 (3.52) nm; IR (KBr) ν_{max} 3252, 2863, 2688, 1600, 1538, 1504, 1472, 1449, 1382, 1338, 1256, 1220, 1121, 1020, 916, 876, 737, 696 cm^{-1}; ^{1}H NMR (CD_3OD, 270 MHz) δ 5.20 (2H, s), 6.93–7.09 (4H, m), 7.31–7.49 (5H, m); ^{1}H NMR ($(CD_3)_2CO$, 270 MHz) δ 5.18 (2H, s), 6.85–6.97 (2H, m), 7.08 (1H, dd, J = 7.8, 1.6 Hz), 7.20 (1H, dd, J = 7.6, 1.9 Hz), 7.33–7.43 (3H, m), 7.55 (2H, d, J = 6.5 Hz); ^{13}C NMR (CD_3OD, 67.5 MHz) δ 71.7, 114.0, 116.9, 122.4, 125.2, 128.7, 129.2, 129.6, 135.1, 138.1, 149.5; HRESIMS m/z 237.1003 $[M+Na]^+$ (calcd for $C_{13}H_{14}N_2ONa$, 237.1004).
17. *N^2-(Benzyloxycarbonyl)-L-glutamic acid 1-(benzyl ester) 5-[(2-benzyloxy-phenyl)-hydrazide]* (**11**): *Z*-Glu-OBn **7** (407 mg, 1.1 mmol), hydrazine hydrochloride **8** (318 mg, 1.3 mmol, 1.2 equiv) and TBTU (528 mg, 1.6 mmol, 1.5 equiv) were dissolved in DMF (8 mL), and the solution was cooled at −15 °C. DIPEA (0.570 mL, 3.3 mmol, 3 equiv) was added and the mixture stirred for 2 h at room temperature. The reaction mixture was then poured into 1 M $NaHSO_4$ (60 mL) and extracted with EtOAc (150 mL). The organic layer was washed consecutively with 1 M $NaHSO_4$ (60 mL), aqueous $NaHCO_3$ (3 × 40 mL), and aqueous NaCl (40 mL). After drying (Na_2SO_4), the solvent was evaporated under reduced pressure. The residue was filtrated on silica, using CH_2Cl_2/MeOH (95:5) as eluent, evaporated again, and finally triturated in Et_2O, to provide hydrazide **11** as a white precipitate (436 mg, 71%): mp 151–153 °C; $[\alpha]_D^{20}$ −1 (*c* 1, $CHCl_3$); UV (CH_2Cl_2) λ_{max} (log ε) 237 (3.81), 284 (3.33) nm; IR (KBr) ν_{max} 3293, 3032, 1731, 1688, 1651, 1600, 1538, 1503, 1447, 1378, 1345, 1305, 1262, 1238, 1208, 1130, 1064, 1018, 977, 907, 862, 737, 697, 477 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz, 60 °C) δ 1.87 (1H, m), 2.06 (1H, m), 2.27 (2H, m), 4.15 (1H, m), 4.98 (2H, s), 5.05 (2H, s), 5.14 (2H, s), 6.65 (2H, d, J = 8.8 Hz), 6.80 (2H, d, J = 8.8 Hz), 7.29–7.41 (15H, m), 7.65 (1H, d, J = 4.5 Hz), 9.47 (1H, s); ^{13}C NMR (DMSO-d_6, 500 MHz, 60 °C) δ 26.0, 29.2, 53.2, 65.1, 65.5, 69.3, 111.5, 111.8, 120.6, 120.6, 126.9, 127.2, 127.2, 127.3, 127.5, 127.8, 127.9, 135.4, 136.4, 136.7, 138.1, 145.0, 170.3, 171.4; HRESIMS m/z 590.2270 $[M+Na]^+$ (calcd for $C_{33}H_{33}N_3O_6Na$, 590.2267).
N^2-(Benzyloxycarbonyl)-L-glutamic acid 1-(benzyl ester) 5-[(3-benzyloxy-phenyl)-hydrazide] (**12**): The peptide-bond-forming reaction described above (see **11**) was employed using (3-benzyloxy-phenyl)-hydrazine hydrochloride **9** (242 mg, 0.97 mmol), *Z*-Glu-OBn **7** (299 mg, 0.81 mmol), TBTU (390 mg, 1.22 mmol) and DIPEA (0.420 mL, 2.5 mmol), in anhydrous DMF (5 mL). Reaction went to completion in 2 h. The reaction mixture was then poured into 1 M $NaHSO_4$ (30 mL) and extracted with EtOAc (30 mL). The organic layer was washed consecutively with 1 M $NaHSO_4$ (30 mL), aqueous $NaHCO_3$ (3 × 20 mL), and aqueous NaCl (20 mL). After drying (Na_2SO_4), the solvent was evaporated under reduced pressure. The residue was filtrated on silica, using CH_2Cl_2/MeOH (95:5) as eluent, evaporated again, and finally triturated in Et_2O, to provide hydrazide **12** as a white precipitate (343 mg, 76%): mp 109–11 °C; $[\alpha]_D^{20}$ −4 (*c* 1, $CHCl_3$); UV (CH_2Cl_2) λ_{max} (log ε) 232 (3.95), 283 (3.43) nm; IR (KBr) ν_{max} 3312, 3032, 1745, 1691, 1645, 1600, 1538, 1496, 1452, 1341, 1269, 1186, 1153, 1056, 1003, 826, 736, 695 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz, 60 °C) δ 1.86–2.31 (4H, m), 4.17 (1H, m), 5.02 (2H, s), 5.04 (2H, s), 5.14 (2H, s), 6.31–6.36 (3H, m), 7.00 (1H, m), 7.31–7.42 (15H, m), 7.50 (1H, s), 9.48 (1H, s); ^{13}C NMR (DMSO-d_6, 500 MHz, 60 °C) δ 26.1, 29.1, 53.2, 65.1, 65.5, 68.7, 98.6, 104.4, 104.8, 126.9, 127.1, 127.1, 127.2, 127.4, 127.8, 127.8, 127.8, 128.0, 128.9, 135.4, 136.4, 136.9, 150.3, 155.6, 158.9, 170.5, 171.4; HRESIMS m/z 590.2269 $[M+Na]^+$ (calcd for $C_{33}H_{33}N_3O_6Na$, 590.2267).
N^2-(Benzyloxycarbonyl)-L-glutamic acid 1-(benzyl ester) 5-[(4-benzyloxy-phenyl)-hydrazide] (**13**): The peptide-bond-forming reaction described above (see **11**) was employed using (4-benzyloxy-phenyl)-hydrazine hydrochloride **10** (318 mg, 1.29 mmol), *Z*-Glu-OBn **7** (407 mg, 1.09 mmol), TBTU (528 mg, 1.64 mmol) and DIPEA (0.570 mL, 3.4 mmol), in anhydrous DMF (8 mL). Reaction went to completion in 2 h and was treated as described above (see **11**), to provide hydrazide **13** as a white precipitate (436 mg, 71%): mp 163–165 °C; $[\alpha]_D^{20}$ −5 (*c* 1, $CHCl_3$); UV (CH_2Cl_2) λ_{max} (log ε) 238 (4.12), 298 (3.35) nm; IR (KBr) ν_{max} 3295, 3033, 1728, 1688, 1629, 1543, 1507, 1453, 1380, 1276, 1236, 1171, 1063, 824, 747, 695 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz, 60 °C) δ 1.90 (1H, m), 2.08 (1H, m), 2.31 (2H, m), 4.17 (1H, m), 5.06 (2H, s), 5.15 (4H, s), 6.71–6.81 (4H, m), 6.95 (1H, d, J = 7.3 Hz), 7.32–7.38 (13H, m), 7.50 (2H, d, J = 6.6 Hz), 7.66 (1H, s), 9.67 (1H, s); ^{13}C NMR (DMSO-d_6, 500 MHz, 60 °C) δ 26.1, 29.1, 53.2, 65.1, 65.5, 69.5, 113.2, 115.0, 126.9, 127.0, 127.1, 127.2, 127.2, 127.5, 127.8, 127.9, 127.9, 128.0, 135.5, 136.4, 137.2, 143.0, 151.3, 170.5, 172.4; HRESIMS m/z 590.2262 $[M+Na]^+$ (calcd for $C_{33}H_{33}N_3O_6Na$, 590.2267).
18. *L-Glutamic acid 5-[(2-hydroxyphenyl)-hydrazide]* (**4**): A catalytic quantity of 10% Pd/C (about 10 mg) were added to a suspension of hydrazide **11** (482 mg, 0.85 mmol) in 40 mL of MeOH. H_2 was applied from a balloon on the top of the flask with stirring for 5 h. Then, the mixture was filtered, the filtrate evaporated to yield hydrazide **4** as a pale yellow powder (225 mg, 89%): mp (degradation) 144–146 °C; $[\alpha]_D^{20}$ −2 (*c* 1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.31), 233 (3.82), 282 (3.43) nm; IR (KBr) ν_{max} 3039, 2609, 1659, 1650, 1644, 1633, 1613, 1557, 1537, 1504, 1455, 1392, 1247, 1201, 748, 666, 542, 469 cm^{-1}; ^{1}H NMR (D_2O, 270 MHz) δ 2.17 (2H, m), 2.50 (2H, m), 3.77 (1H, t, J = 6.2 Hz), 6.86 (4H, s); ^{13}C NMR (D_2O, 67.5 MHz) δ 26.7, 30.1, 54.7, 114.4, 116.0, 121.6, 122.3, 136.1, 144.4, 174.4, 175.2; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.92 (2H, m), 2.36 (2H, m), 3.28 (1H, t, J = 6.3 Hz), 6.58 (1H, m), 6.64 (2H, m), 6.71 (1H, s), 6.73 (1H, ps d, J = 7.1 Hz), 10.02 (2H, br s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 27.0, 29.9, 53.5, 112.2, 114.2, 119.1, 119.1, 137.2, 144.3, 169.9, 171.2; HRESIMS m/z 252.0987 $[M-H]^-$ (calcd for $C_{11}H_{14}N_3O_4$, 252.0984).
L-Glutamic acid 5-[(3-hydroxyphenyl)-hydrazide] (**5**): A catalytic quantity of 10% Pd/C (about 10 mg) were added to a suspension of hydrazide **12** (210 mg, 0.37 mmol) in 15 mL of MeOH. H_2 was applied from a balloon on the top of the flask with stirring for 4 h. Then, the mixture was filtered, the filtrate evaporated to yield hydrazide **5** as a pale yellow powder (59 mg, 63%): mp (degradation) 173–174 °C; IR (KBr) ν_{max} 3242, 3053, 2601, 2125, 1681, 1659, 1651, 1643, 1633, 1605, 1567, 1556, 1537, 1519, 1504, 1454, 1415, 1153, 993, 766, 689, 542, 483, 457 cm^{-1}; ^{1}H NMR (D_2O, 270 MHz) δ 2.17 (2H, m), 2.50 (2H, m), 3.78 (1H, t, J = 6.2 Hz), 6.36 (1H, d, J = 2.2 Hz), 6.44 (2H, m), 7.15 (1H, t, J = 8.1 Hz); ^{13}C NMR (D_2O, 67.5 MHz) δ 27.6, 30.9, 55.6, 101.7, 107.1, 109.5, 132.2, 150.5, 158.1, 175.2, 176.1; HRESIMS m/z 252.0986 $[M-H]^-$ (calcd for $C_{11}H_{14}N_3O_4$, 252.0984).
L-Glutamic acid 5-[(4-hydroxyphenyl)-hydrazide] (**6**): A catalytic quantity of 10% Pd/C (about 10 mg) were added to a suspension of hydrazide **13** (384 mg, 0.68 mmol) in 40 mL of MeOH. H_2 was applied from a balloon on the top of the flask with stirring for 4 h. Then, the mixture was filtered, the filtrate evaporated to yield hydrazide **6** as a pale yellow powder (160 mg, 93%): mp (degradation) 190–192 °C; IR (KBr) ν_{max} 3266, 3024, 2601, 2101, 1651, 1583, 1557, 1513, 1505, 1408, 1233, 978, 824, 668, 514 cm^{-1}; ^{1}H NMR (D_2O, 270 MHz) δ 2.17 (2H, m), 2.49 (2H, m), 3.77 (1H, t, J = 6.2 Hz), 6.81 (4H, s); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 1.90 (2H, m), 2.30 (2H, m), 3.23 (1H, t, J = 6.2 Hz), 6.51–6.63 (4H, ps s), 7.12 (1H, s), 9.81 (1H, s); ^{13}C NMR (DMSO-d_6, 67.5 MHz) δ 27.1, 30.0, 53.6, 113.9, 115.3, 141.7, 150.4, 169.8, 171.6; HRESIMS m/z 252.0991 $[M-H]^-$ (calcd for $C_{11}H_{14}N_3O_4$, 252.0984).
19. HPLC experiments were performed on a chiral column (Chiralpak® AD-H, Chiral technologies Europe, Illkirch, France). Each sample was diluted in ethanol at a concentration of 0.1 mg/mL, and after passing through a 0.45-μm membrane filter, 20 μL were injected into the column, using heptane/isopropanol (60:40) as an eluent, with a flow rate of 0.7 mL/min. Peak detection was carried out online using a UV detector at 254 nm. The retention time of **3** and **4** was 6.13 min.

20. Rueppel, M. L.; Lord Lundt, S.; Gass, J. D.; Meister, A. *Biochemistry* **1972**, *11*, 2839.
21. Cytotoxic activity of compounds was determined for B16 (murine melanoma cells), and A375 (human melanoma cells), by the neutral red uptake (NRU) assay based on the initial protocol described by Borefreund and Puerner (1985).[24] Briefly, 96-well tissue culture plates were seeded with B16 and A375 at, respectively, 10×10^3 and 12×10^3 cells/well. The plates were then incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. Cells were then exposed to dilutions of the test compounds in RPMI medium for 24 and 48 h. Etoposide was used as a positive control and cell viability was assessed using the NRU assay. It consisted of a 3 h-incubation with neutral red (50 μg mL^{-1} in RPMI) followed by extraction with a mixture (150 μL) ethanol/acetic acid/water (50:1:49). The absorbance was measured at 540 nm using a microplate spectrophotometer system. Results are presented as percentage of control values and IC_{50} values were determined graphically from concentration-effect curves of at least three experiments.
22. Boekelheide, K.; Graham, D. G.; Mize, P. D.; Koo, E. H. *J. Invest. Dermatol.* **1980**, *75*, 322.
23. Gohil, V. M.; Agrawal, S. K.; Saxena, A. K.; Garg, D.; Gopimohan, C.; Bhutani, K. K. *Indian J. Exp. Biol.* **2010**, *48*, 265.
24. Borenfreund, E.; Puerner, J. A. *Toxicol. Lett.* **1985**, *24*, 119.