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A Simple Procedure for Preparation of *N*-Thiazolyl and *N*-Thiadiazolylcantharidinimides and Evaluation of Their Cytotoxicities against Human Hepatocellular Carcinoma Cells

Pen-Yuan Lin,* Sheng-Jie Shi,* Hsien-Liang Shu,* Hsue-Fen Chen,* Chiung-Chang Lin,* Pong-Chun Liu,* and Leng-Fang Wang†

**School of Pharmacy and †School of Medicine, Department of Biochemistry, Taipei Medical University, 250 Wu-Shing Street, Taipei, Taiwan*

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We made an effort to prepare effective cantharidinimides by heating the reactants **1** and **2a–j** to 200°C with toluene and triethylamine to provide 10 *N*-thiazolyl- and *N*-thiadiazolylcantharidinimides **3a–j** in high yields of 48–91%. All of the synthetic compounds were tested for their capability to suppress growth of the human hepatocellular carcinoma cell lines, SK-Hep-1 and Hep 3B. The results showed that compound **3f** was the most potent, and it was more cytotoxic than cantharidin.

Key Words: cantharidin; *N*-thiazolylcantharidinimide; *N*-thiadiazolylcantharidinimide; human hepatocellular carcinoma cell; cytotoxicity.

INTRODUCTION

Cantharidin **1** is found in *Mylabris caraganae* and various other insects. In clinical studies it has been shown to possess antitumor and antihepatoma properties. It is reported to have extremely high potency as well as showing toxic properties (*1–3*), which makes it useless in the clinic. It is used as a standard in research confined to vetertinary medicine due to it's irritant and vesicating effects. In a search for less toxic analogues of cantharidin or cantharidinimide derivatives, a slightly modified structure has been synthesized in an analogous manner (*4*). Cantharidin **1** can undergo a ring-opening reaction to become dicarboxylic acid and can be prepared as a series of imides by heating with primary amine. The formation of products of the *N*-aliphatic imides is more rapid than that of aromatic imides (*5*). The present study shows that the characters of amine basicity and chosen temperature are crucial, and the characters of the group and their position on the aromatic ring also influence yields. In order to obtain novel types of related imides and to study the scope of these synthetic reactions, the same technique was applied to the reaction of compound **1** with thiazolylamine or thiadiazolylamine in a high-pressure tube with dry toluene and TEA (Triethylamine) heated to ca. 200°C. This method gave good yields after evaporation and

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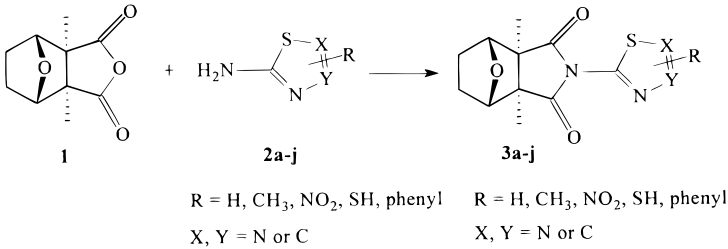


SCHEME 1.

purification by silica gel column chromatography and recrystallization in methanol. (Scheme 1).

RESULTS AND DISCUSSION

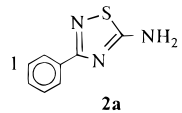
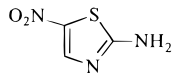
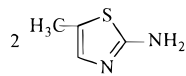
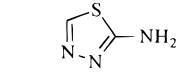
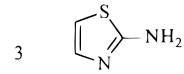
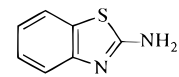
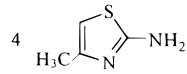
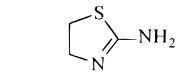
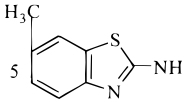
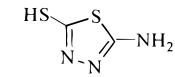
As shown in Table 1, the *N*-thiazolyl- and *N*-thiadiazolylcantharidinimides **3a–3j** could be prepared by means of the pressure technique synthesis. The yields vary from 48% to 91% and show a trend compatible with expected basicity, and characters of the thiazolyl and thiadiazolyl ring groups influencing compound **2**. High yields were obtained for **3a** to **3d**. The NH_2 basicities of aminothiazolylcantharidinimides and aminothiadiazolylcantharidinimides are unknown but will be slightly different between one of corresponding aminothiazols which has an electron deficiency of the thiazol and thiadiazol rings. Variations in yields of **3a, 3g**, and **3j** may perhaps reflect inductive electron donation and electron withdrawal by the thiadiazolyl ring, since an inductive effect will inversely increase with distance between the three nitrogen atoms and sulfur atom. The results obtained with **3b, 3e, 3d, 3e, 3f**, and **3h**, however, strongly confirm the influence of amine nucleophilicity and their basicities, and the characters of functional group position on the ring. Compound **2f** exerted the most electron-withdrawing capability with resonance and induction effects, and the formation of cantharidinimide appeared to become more difficult. It should be noted that the more conjugated character, the higher the yield that would be obtained, as is seen in **3c** > **3i**. The preparative technique was also influenced by other factors that can cause strong variations in the results. The formation of cantharidinimides might be expected via ring opening and dehydrated reaction steps and hence the reaction temperature was also a crucial factor in this formation.

The potential cytotoxicity of the prepared cantharidinimides was investigated against hepatocellular carcinoma cell lines, Hep 3B (*6*) and SK-Hep-1 (*7*) and evaluated using MTT cell viability assays (Table 2). It has been shown that viable cell numbers correlate with optical density as determined by the MTT assay (*8,9*).

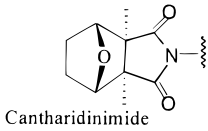
Being comparable in cytotoxicity to cantharidin, the IC_{50} values of all of the cantharidinimide derivatives (**3a–j**) were 0.6 to 900 μM, and of cantharidin were 2 to 4 μM and of **C-N** (Cantharidinimide) and **C-M** (*N*-Methylcantharidinimide) were completely inactive up to the highest concentration tested (2000 μM). Since **C-M** has been produced as an antihepatoma drug in China (*10*), the reason that it was noncytotoxic to the tested hepatoma cell lines was unknown. The lack of activity for

TABLE 1

Preparation of Cantharidinimide Derivatives (**3a–3j**)[a]

1		**2a**	**3a** (91)	6		**2f**	**3f** (69)
2		**2b**	**3b** (89)	7		**2g**	**3g** (60)
3		**2c**	**3c** (87)	8		**2h**	**3h** (55)
4		**2d**	**3d** (86)	9		**2i**	**3i** (54)
5		**2e**	**3e** (69)	10		**2j**	**3j** (48)

[a] **3a–3j**: Cantharidinimides.
[b] **2a–2j**: Amines.
[c] The yields obtained after purification by chromatography on silical gel.



C-N suggested that the presence of a thiazole or thiadiazole moiety is probably important for the cytotoxic properties of this series. The IC_{50} values of thiazolylcantharidinimides decreased in the order **3b** > **3d** ≈ **3e** > **3c** > **3h** ≈ **3i** > **3f**. In this study, the only compound showing higher cytotoxicity than cantharidin was **3f** in which a nitrosubstituent was introduced on the 5′-position of thiazole group of **3c**; while compound with a methyl substituent at 5′- or 4′-position of thiazole group of **3c** reduced the cytotoxic activity and the position of methyl- also affected the biological activity, it produced three- to five-fold difference effects on the cell (**3b** vs **3d**). The saturation of the 4′-, 5′-double bond of thiazole group led to a four-fold increased in cytotoxicity against tumor cell lines tested (**3i** vs **3c**). The result showed that the presence of electron withdrawing substituents (**3h**, **3i**, and **3f**) markedly enhanced cytotoxicity (**3b**, **3d**, and **3e**).

TABLE 2

Cytotoxicity of Cantharidin **1**, C-N, C-M, *N*-thiazolyl-, and *N*-Thiadiazolylcantharidinimides in Human Hepatocellular Carcinoma Cell Lines

	IC_{50} (μM)[a]												
Cell line	**1**	**C-N**[b]	**C-M**[c]	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**	**3h**	**3i**	**3j**
Hep-3B	2	>2000	>2000	56	360	57	130	ND[d]	0.4	22	8	11.2	14.4
SK-Hep-1	4	>2000	>2000	48	900	51	180	110	1.25	56	14	13	16

[a] IC_{50} was calculated after 48 h of continuous drug exposure, values are means of three to four experiments with coefficients of variation of 5–10%.

[b] Cantharidinimide.

[c] *N*-methylcantharidinimide.

[d] Not determined.

Furthermore, **3h** displayed higher cytotoxicity and less electronegativity than that of **3e**. It can be concluded that the increase with the electronegativity of the substituent group will decrease the cytotoxicity. The IC_{50} values of thiadiazolylcantharidinimides decreased in the order **3a** $\approx$ **3g** $>$ **3j**. A thiol substituent on thiadiazole enhanced the biological activity (**3j** vs **3g** and **3j** vs **3a**). The result also showed that the electronegativity of the substituent group play an important role on the cytotoxicity.

EXPERIMENTAL

Chemistry

Infrared spectra were recorded on a Perkin–Elmer Model 882 and a Nicolet 510 PET spectrophotometers. ^{1}H NMR spectra ($CDCl_3$ unless otherwise stated) were recorded at 300 MHz on a Bruker AC and at 400 MHz on a Bruker AC and at 500 MHz on a Bruker Advance DRX. Melting points were determined by a Yanaco MP-S_3 melting point apparatus. Mass spectra were obtained on a Joel JMSHX 110 FABMS spectrometer; elemental analysis spectra were obtained on a Perkin–Elmer 2400. The tube was Büchi glasuster (Bursting disc, 0032). General procedures were followed for the reaction of compound **2** with cantharidin.

These compounds were prepared according to similar procedure and reactions took place in high-pressure tubes. Cantharidin was added to a tube containing 3 ml of dried toluene and triethylamine; the solution was stirred and heated to ca. 200°C. After being stirred for 2 h, the mixture was evaporated, and the residue mass was purified by column chromatography and recrystallized from methanol.

Antineoplastic Bioassays

Cell culture. Media and sera for cell culture were purchased from Life Technologies, Inc. Most chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). SK-Hep-1 and Hep-3B, the human hepatocarcinoma cells lines obtained from American Type Culture Collection (ATCC) (Rockville, MD), were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated

fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids and 1 mM glutamine in a controlled atmosphere of 5% CO_2, 95% air at 37°C.

MTT assay for cellular viability. Cells were seeded into 96-well plates and allowed to adhere for 24 h before drugs were introduced. Following a 48-h incubation, drugs and medium were removed by flicking and each well was treated with 100 μl of 500 μg/ml MTT [3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide] in culture medium. Following a 4-h incubation period to allow metabolism of MTT by mitochondrial dehydrogenases of viable cells to form an insoluble formazan product, the crystals were dissolved in 100 μl of acid-SDS (0.01 N HCL in 10% SDS) by incubating the plates overnight. Absorbance, as a measure of viable cell number, was read the following day in a model MA310 automated EIA plate reader at a wavelength of 550 nm. IC_{50} values were obtained by a linear regression analysis of percentage absorbance versus log drug concentration.

*N-[5-(3-Phenyl-1,2,4-thiadiazolyl)]cantharidinimides (***3a**)

mp 207–208°C (MeOH); ^{1}HNMR (500 MHz, $CDCl_3$): δ(ppm) 1.32 (s, 6H, CH_3 × 2), 4.77 (d, 2H, *J* 2.2 Hz, OCH), 7.46 (mc, 1H, phenyl H-4′), 7.47 (m, 1H, phenyl-H-3′), 7.48 (m, 1H, phenyl H-5′), 8.34 (d, 1H, *J* 3.8 Hz, phenyl H-2′), 8.35 (d, 1H, *J* 3.8 Hz, phenyl H-6′); IR (KBr) 1715 (amide) cm^{-1}; MS *m/z* (rel int): 355 $[M]^+$, (35), 286 (100), 135 (80); HRMS (EI, 80 ev) calcd for $C_{18}H_{15}N_3O_3S$: 355.0991. Found: 355.0976.

*N-[2-(5-Methylthiazolyl)]cantharidinimide (***3b**)

mp 150–152 °C (MeOH); ^{1}HNMR (300 MHz, $CDCl_3$): δ(ppm) 1.26 (s, 6H, CH_3 × 2), 1.72–1.86 (m, 4H, CH_2×2), 2.17 (s, 3H, CH_3), 4.72 (t, 2H, *J* 2.2 Hz, OCH), 7.42 (s, 1H, thiazol ring H-3′); IR (KBr): 1725 (amide) cm^{-1}, MS m/z (rel int) 292 $[M]^+$, (25), 223 (100); HRMS (EI, 80 ev) calcd for $C_{14}H_{16}N_2O_3S$: 292.0855. Found: 292.0874.

*N-(2-Thiazolyl)cantharidinimide (***3c**)

mp 174–175°C (MeOH); ^{1}HNMR (300 MHz, $CDCl_3$): δ(ppm) 1.28 (s, 6H, CH_3 × 2), 1.74–1.88 (m, 4H, CH_2 × 2), 4.74 (t, 2H, *J* 2.5 Hz, OCH), 7.33 (d, 1H, *J* 3.6 Hz, thiazolyl H-4′), 7.78 (d, 1H, *J* 3.5 Hz thiazol H-3); IR (KBr): 1724 (amide) cm^{-1}, MS *m/z* (rel int) 278 $[M]^+$, (15), 209 (100); HRMS (EI, 80 ev) calcd for $C_{13}H_{14}N_2O_3S$: 278.0725. Found: 278.0729.

*N-[2-(4-Methylthiazolyl)]cantharidinimide (***3d**)

mp 167–169°C (MeOH); ^{1}HNMR (500 MHz, $CDCl_3$): δ(ppm) 1.27 (s, 6H, CH_3 × 2), 1.67–2.24 (m, 4H, CH_2 × 2), 2.39 (s, 3H, CH_3), 4.59 (s, 2H, OCH), 7.11 (s, 1H, thiazol H-3′); IR (KBr): 1714 (amide) cm^{-1}, MS *m/z* (rel int) 292 $[M]^+$, (15), 223 (100), 96 (35); HRMS (EI, 80 ev) calcd for $C_{14}H_{16}N_2$ O_3S: 292.0882. Found: 292.0880.

*N-[4-Phenyl-(6-methylbenzothiazolyl)]cantharidinimide (***3e**)

mp 202–205°C (MeOH); ^{1}HNMR (400 MHz, $CDCl_3$): δ(ppm) 1.27 (s, 6H, CH_3 × 2), 1.76–1.88 (m, 4H, CH_2 × 2), 2.50 (s, 3H, CH_3), 4.71 (d, 2H, *J* 2.0 Hz, OCH),

7.32 (d, 1H, *J* 8.8 Hz, H-5), 7.69 (s, 1H, benzothiazoly H-7), 8.0 (d, 1H, *J* 8.0 Hz, benzothiazoly H-4); IR (KBr): 1709 (amide) cm^{-1}, MS *m/z* (ret, int.): 418 (M^+, 90), 349 (70), 121 (70), 96 (100); HRMS (EI, 80 ev) calcd for $C_{24}H_{22}N_2O_3S$: 418.1351. Found: 418.1313.

*N-[2-(5-Nitrothiazolyl)]cantharidinimide (***3f**)

mp 212–214°C (MeOH); ^{1}HNMR (400 MHz, $CDCl_3$): δ(ppm) 1.27 (s, 6H, CH_3 × 2), 1.70–1.90 (m, 4H, CH_2 × 2), 4.65 (t, 2H, *J* 2.4 OCH), 7.69 (s, 1H, thiazolyl H-4); IR (KBr): 1780 (amide) cm^{-1}, MS *m/z* (rel int): 323 $[M]^+$, (5), 128 (90), 96 (100).

*N-[2-(1,3,4-Thiadizolyl)]cantharidinimide (***3g**)

mp 133–134°C (MeOH); ^{1}HNMR (400 MHz, $CDCl_3$): δ(ppm) 1.31 (s, 6H, CH_3 × 2), 1.77–1.89 (m, 4H, CH_2 × 2), 4.76 (s, 2H, OCH), 9.12 (s, 1H,thiadizolyl H-5); IR (KBr): 1725 (amide) cm^{-1}; MS *m/z* (rel int): 279 (M^+, 5), 210 (100), 128 (40), HRMS (EI, 80 ev) calcd for $C_{12}H_{13}N_3O_3S$: 279.0678. Found: 279.0744.

*N-(2-Benzothiazolyl)cantharidinimide (***3h**)

mp 165–167°C (MeOH); ^{1}HNMR (300 MHz, $CDCl_3$): δ(ppm) 1.30 (s, 6H, CH_3 × 2), 1.74–1.92 (m, 4H, CH_2 × 2), 4.77 (t, 2H, *J* 2.4 Hz OCH), 7.43 (dd, 1H, *J* 7.6 Hz; *J* 14.6 Hz H-6′), 7.49 (dd, 1H, *J* 7.5 Hz; *J* 14.4 Hz, H-5′), 7.89 (d, 1H, *J* 7.9 Hz H-7′), 8.12 (d, 1H, *J* 7.9 Hz, H-4′); IR (KBr): 1725 (amide) cm^{-1}, MS *m/z* (rel int): 328 $[M]^+$, (30), 259 (100), 96 (80), 67 (90); HRMS (EI, 80 ev) calcd for $C_{17}H_{16}N_2O_3S$: 328.0882. Found: 328.0908.

*N-(2-Thiazolyl)cantharidinimide (***3i**)

mp 197–199°C (MeOH); ^{1}HNMR (500 MHz, $CDCl_3$): δ(ppm) 1.12 (s, 6H, CH_3 × 2), 1.21 (2H, d, *J* 5.1Hz, SCH_2). 1.67–1.78 (m, 4H, CH_2 × 2), 2.00 (d, 2H, *J* 5.0 Hz, NCH_2), 4.58 (t, 2H, *J* 2.5Hz, OCH); IR (KBr):1703 (amide)cm^{-1}, MS *m/z* (rel int): 280 (M^+, 5), 195 (20), 127 (100), 96 (68); HRMS (EI, 80 eV) calcd for $C_{13}H_{16}N_2O_3S$: 280.3503. Found: 280.3516.

*N-[2-(5-Mecapto-1,3,4-thiadiazolyl)]cantharidinimide (***3j**)

mp 213–215°C (MeOH), ^{1}HNMR (500MHz, $CDCl_3$): δ(ppm) 1.20 (s, 6H, CH_3 × 2), 1.70–l.90 (m, 4H, CH_2 × 2), 4.73 (t, 2H, *J* 2.3 Hz, OCH). 9.14 (s, 1H, SH); IR (KBr): 1708 (amide) cm^{-1}, MS *m/z* (rel int) 311 (M^+, 10), 96 (100), 128 (60), 70 (50); HRMS (EI, 80 eV) calcd for $C_{12}H_{13}N_3O_3S_2$: 311.0398. Found: 311.0389.

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