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Studies of the Constituents of Uruguayan Propolis

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Eighteen flavonoids including two new compounds, four aromatic carboxylic acids, and eleven phenolic acid esters including one new compound were isolated and identified from the ethyl acetate soluble fraction of the 70% ethanol extract of Uruguayan propolis. The new compounds were elucidated as pinobanksin 3-(2-methyl)butyrate (**1**; recently reported in Usia, T.; Banskota, A. H.; Tezuka, Y.; Midorikawa, K.; Matsushige, K.; Kadota, S. *J. Nat. Prod.* **2002**, *65*, 673−676) pinobanksin 3-isobutyrate (**2**), and 2-methyl-2-butenyl ferulate (**24**). The constituents isolated from Uruguayan propolis in this study were similar to those of propolis of European and Chinese origin. Thus, it is suggested that the Uruguayan propolis has a plant origin similar to those of propolis from Europe and China.

KEYWORDS: Propolis; Uruguay; flavonoid; phenolics; poplar

INTRODUCTION

Propolis, or bee glue, a natural resinous hive product gathered by honeybees from buds and exudates of certain trees and plants, has been considered as a protective barrier against their enemies. Propolis has been used as a folk medicine in many regions of the world (*1*). Propolis has been reported to have various biological activities such as antibacterial (*2*), antiviral (*3*), fungicidal (*4*), antiinflammatory (*5*), and anticancer (*6*) properties.

The chemical composition of propolis is extremely complex, and more than 180 constituents have been identified so far, the most important ones being polyphenols (*7*). The constituents, and various biological and pharmacological activities, of propolis from Brazil, China, and Europe have been reported (*8*−*30*), but the detailed constituents of Uruguayan propolis have not been reported. Bonvehí et al. (*31*) have reported the antibacterial and radical scavenging activity of Uruguayan propolis, and identified several compounds in it. However, their study to identify the compounds was based on the HPLC analysis alone and was not based on MS and NMR analysis. Thus, we investigated the constituents of Uruguayan propolis using MS and NMR techniques after isolation of each compound. Bonvehí et al. (*31*) further have described that the origin plants of Uruguayan propolis are *Eucalyptus globules*, *Populus* sp., *Betula* sp., and *Salix* sp. We compared the isolated compounds with those of the propolis from other geographic origins, and discussed the plant origin of Uruguayan propolis.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were determined with a Jasco DIP-1000 digital polarimeter. UV spectra were measured on a Hitachi U2000 spectrophotometer. IR spectra were measured on a Jasco FT/IR-550 Fourier transform IR spectrometer. Circular dichroism (CD) spectra were recorded on a Jasco J-600 spectrometer. FAB-MS spectra were taken on a JEOL JMS-700 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-α400 (400 and 100 MHz, respectively) spectrometer using TMS as an internal standard. Chemical shifts are given as δ values (ppm) and coupling constants (J) are given in Hertz (Hz). The ^{1}H−^{1}H COSY, HSQC, and HMBC spectra were recorded with standard JEOL software. Acetone-d_6 and CD_3OD were obtained from EURISO-TOP (France). DMSO-d_6 was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Isolation of Constituents from Propolis. Propolis collected in Uruguay was supplied from Aichi Uruguay S. A. (Montevideo, Uruguay). Dried propolis sample (50 g) was extracted with 250 mL of 70% EtOH at room temperature for 2 h with stirring. The 70% EtOH extract was concentrated under reduced pressure and delipidated with hexane (300 mL) to give a crude material. This was partitioned with EtOAc−H_2O to yield an EtOAc extract (25.17 g) and H_2O extract. The EtOAc extract was subjected to silica gel column chromatography (50 × 300 mm i.d.) with an EtOAc/MeOH gradient system to give 27 fractions: frs. 1−11, EtOAc eluate; frs. 12−17, EtOAc/MeOH (8:2) eluate; frs. 18−23, EtOAc/MeOH (1:1) eluate; and frs. 24−27, MeOH eluate. Each fraction was collected by 100 mL. Fraction 2 was rechromatographed by preparative HPLC on a 20 × 250 mm i.d. ODS column (Capcell Pak UG-120, Shiseido, Japan) with 0.1% trifluoroacetic acid (TFA) in CH_3CN−H_2O (4:6 or 5:5) at 8 mL/min to give compounds **1** (8.4 mg), **2** (2.6 mg), **3** (4.9 mg), **4** (4.2 mg), **5** (11.6 mg), **6** (11.9 mg), **7** (6.8 mg), **8** (2.9 mg), **9** (15.6 mg), **10** (10.6 mg), **11** (18.2 mg), **13** (45.2 mg), **14** (3.0 mg), **15** (14.3 mg), **16** (19.1 mg), **17** (2.4 mg), **18** (1.0 mg), **23** (2.5 mg), **24** (5.5 mg), **25** (3.2 mg), **26** (9.9 mg), **27** (1.2 mg), **29** (5.1 mg), **30** (19.7 mg), **32** (4.1 mg), and **33** (1.0 mg). Fraction 12 was repeatedly rechromatographed by preparative HPLC on a 20 × 250 mm i.d. ODS column (Capcell Pak UG-120, Shiseido, Japan) with 0.1% TFA in CH_3CN−H_2O (3:7) at 8 mL/min to give compounds **12** (3.3 mg), **21** (2.3 mg), **22** (3.2 mg), **28** (2.0 mg), and **31** (9.7 mg). Compounds **19** (2.0 mg) and **20** (1.2 mg) were isolated from fraction 22 by preparative HPLC on a 20 × 250 mm i.d. ODS column (Capcell Pak UG-120, Shiseido, Japan) with 0.1% TFA in CH_3CN−H_2O (2:8) at 8 mL/min.

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Table 1. NMR Data of Pinobanksin 3-(2-Methyl)butyrate (**1**) and Pinobanksin 3-Isobutyrate (**2**)[a]

	1[b]		**2**[c]	
	δ_H	δ_C	δ_H	δ_C
2	5.41 d (J = 12.2 Hz)	82.7	5.55 d (J = 12.0 Hz)	82.1
3	5.87 d (J = 12.2 Hz)	73.3	5.90 d (J = 12.0 Hz)	72.8
4		193.1		192.9
5		165.5		165.3
5-OH			11.58 s	
6	5.94 d (J = 2.4 Hz)	96.6	6.05 d (J = 2.0 Hz)	96.4
7		169.0		168.2
7-OH			Not observed	
8	5.96 d (J = 2.4 Hz)	97.8	6.07 d (J = 2.0 Hz)	97.5
9		164.1		163.9
10		102.1		102.4
1′		137.2		136.9
2′	7.52 m	129.0	7.61 dd (J = 7.0, 1.5 Hz)	129.4
3′	7.40 m	129.6	7.46 m	128.8
4′	7.40 m	130.5	7.46 m	130.2
5′	7.40 m	129.6	7.46 m	128.8
6′	7.52 m	129.0	7.61 dd (J = 7.0, 1.5 Hz)	129.4
1″		176.3		175.4
2″	2.31 tq (J = 7.3 Hz)	42.1	2.49 q (J = 6.8 Hz)	34.3
2″-Me	1.01 d (J = 7.3 Hz)	17.0	1.03, 0.91 d (J = 6.8 Hz)	19.0 (x2)
3″	1.43 m	27.6		
4″	0.58 t (J = 7.3 Hz)	11.4		

[a] ^{1}H and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively. [b] Measured in CD_3OD. [c] Measured in DMSO-d_6.

Pinobanksin 3-(2-methyl)butyrate (**1**): colorless gum; $[\alpha]_D^{26}$ +62.3° (*c* 0.1, MeOH). UV (MeOH) λ_{max} (log ϵ) 292 (4.27) nm. IR (KBr) ν_{max} 3400, 2960, 1640, 1180, 1160, 1090 cm^{-1}. CD (MeOH) $[\theta]_{221}$ +53,-300, $[\theta]_{280}$ −25,600, $[\theta]_{320}$ +8,910. ^{1}H and ^{13}C NMR data are shown in **Table 1**. HRFABMS obsd *m*/*z* 357.1251, calcd 357.1338 $[M + H]^+$.

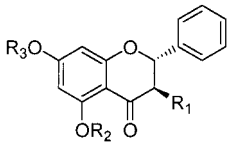
Pinobanksin 3-isobutyrate (**2**): colorless gum; $[\alpha]_D^{26}$ +12.7° (*c* 0.1, MeOH). UV (MeOH) λ_{max} (log ϵ) 293 (3.92) nm. IR (KBr) ν_{max} 3440, 2920, 1690, 1460, 1260, 1160 cm^{-1}. CD (MeOH) $[\theta]_{221}$ +35,700, $[\theta]_{290}$ −18,800, $[\theta]_{325}$ +5,940 .^{1}H and ^{13}C NMR data are shown in **Table 1**. HRFABMS obsd *m*/*z* 343.1146, calcd 343.1182 $[M + H]^+$.

2-methyl-2-butenyl ferulate (**24**): colorless gum. UV (MeOH) λ_{max} (log ϵ) 240 (3.79), 300 (3.98), 327 (4.15) nm. IR (KBr) ν_{max} 3420, 2920, 1700, 1520, 1260, 1160 cm^{-1}. ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.61 (1H, d, J = 16.0 Hz, H-7), 7.35 (1H, d, J = 2.0 Hz, H-2), 7.15 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.88 (1H, d, J = 8.0 Hz, H-5), 6.42 (1H, d, J = 16.0 Hz, H-8), 5.59 (1H, m, H-3′), 4.54 (2H, s, H-1′), 3.92 (3H, s, OC*H*$_3$), 1.68 (3H, s, H-5′), 1.64 (3H, d, J = 6.8 Hz). ^{13}C NMR (acetone-d_6, 100 MHz) δ 167.3 (C-9), 150.0 (C-4), 148.7 (C-3), 145.7 (C-7), 132.2 (C-2′), 127.4 (C-1), 124.0 (C-3′, C-5′), 116.0 (C-5), 115.8 (C-8), 111.3 (C-2), 70.1 (C-1′), 56.3 (OC*H*$_3$), 13.7 (C-4′), 13.3 (C-5′). HRFABMS obsd *m*/*z* 263.1223, calcd 263.1283 $[M + H]^+$.

HPLC Analysis of Propolis. The HPLC system consisted of a Gulliver system (Jasco, Tokyo, Japan) with a Capcell Pak UG120 ODS column (4.6 × 250 mm i.d., Shiseido, Japan). The mobile phase consisted of water with 2% acetic acid (A) and acetonitrile with 2% acetic acid (B). The gradient was 20−80% B in 60 min at a flow rate of 1 mL/min. Chromatograms were recorded at 280 nm. Samples of the standards **1**−**33** were dissolved in methanol (5 mg/mL), and 5-μL aliquots were injected for HPLC analysis. The ethanol extracts of Uruguayan propolis were dissolved in methanol (5 mg/mL), filtered with a 0.45-μm filter (Gelman Sciences, Tokyo, Japan), and aliquots of the filtrate (5 μL) were injected for analysis.

RESULTS AND DISCUSSION

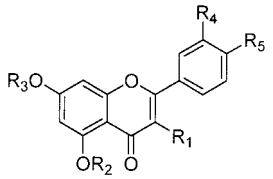
Propolis obtained from Uruguay was extracted with 70% ethanol. The ethyl acetate soluble fraction of the extract was subjected to column chromatographic separation to give eighteen flavonoids, four aromatic carboxylic acids, and eleven phenolic acid esters. Compounds **1**, **2**, and **24** were new compounds. (Compound **1** was recently reported in ref *62*.) **Figure 1** shows the structures of flavanones and flavanonols (**1**−**11**). Compounds **3**−**9** were pinobanksin and its derivatives, pinobanksin 3-hexanoate (*32*) (**3**), pinobanksin 3-butanoate (*25*) (**4**), pinobanksin



	R_1	R_2	R_3
1:	$OCOCH(CH_3)CH_2CH_3$	H	H
2:	$OCOCH(CH_3)_2$	H	H
3:	$OCOC_5H_{11}$	H	H
4:	$OCOC_3H_7$	H	H
5:	$OCOC_2H_5$	H	H
6:	$OCOCH_3$	H	H
7:	$OCOCH_3$	H	CH_3
8:	OH	CH_3	H
9:	OH	H	H
10:	H	H	CH_3
11:	H	H	H

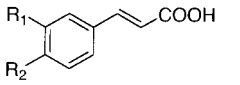
Figure 1. Structures of flavanones and flavanonols isolated from Uruguayan propolis.

3-propanoate (*33*) (**5**), pinobanksin 3-acetate (*34*) (**6**), pinobanksin 3-acetoxy-7-methyl ether (3-acetylalpinone) (*35*) (**7**), pinobanksin 5-methyl ether (*36*) (**8**), and pinobanksin (*34*) (**9**). Compounds **10** and **11** were pinostrobin and pinocembrin, respectively (*33*, *37*). **Figure 2** shows the structures of flavones and flavonols (**12**−**18**). These were chrysin (*34*) (**12**), tectochrysin (*38*) (**13**), chrysin 5-methyl ether (*39*) (**14**), galangin (*34*) (**15**), izalpinin (*40*) (**16**), kaempferol (*41*) (**17**), and quercetin 3-methyl ether (*42*) (**18**). **Figure 3** shows the structures of the aromatic carboxylic acids (**19**−**22**). These were *p*-coumaric acid (**19**), caffeic acid (**20**), 3,4-dimethoxycinnamic acid (*43*) (**21**), and cinnamylideneacetic acid (*36*) (**22**). **Figure 4** shows the structures of phenolic acid esters (**23**−**33**). These were 2-methyl-2-butenyl *p*-coumarate (*44*) (**23**), 3-methyl-3-butenyl ferulate (*45*) (**25**), benzyl *p*-coumarate (*14*) (**26**), benzyl ferulate (*25*) (**27**), phenethyl caffeate (*32*) (**28**), cinnamyl cinnamate (*44*) (**29**), cinnamyl *p*-coumarate (*46*) (**30**), cinnamyl caffeate (*25*) (**31**), cinnamyl isoferulate (*25*) (**32**), and cinnamyl 3,4-dimethoxycinnamate (*47*) (**33**). These known



	R_1	R_2	R_3	R_4	R_5
12:	H	H	H	H	H
13:	H	H	CH_3	H	H
14:	H	CH_3	H	H	H
15:	OH	H	H	H	H
16:	OH	H	CH_3	H	H
17:	OH	H	H	H	OH
18:	OCH_3	H	H	OH	OH

Figure 2. Structures of flavones and flavonols isolated from Uruguayan propolis.



19: R_1, R_2=H
20: R_1=H, R_2=OH
21: R_1, R_2=OCH_3

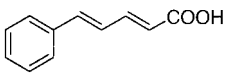


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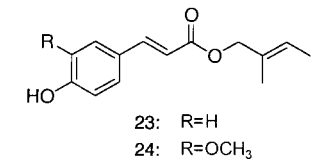
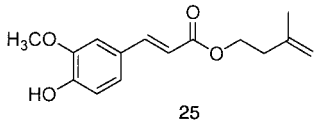
Figure 3. Structures of aromatic carboxylic acids isolated from Uruguayan propolis.

compounds were identified by comparison of spectral data and specific optical rotation with values in the literature and/or authentic samples. This is the first report of the isolation of **7**, **18**, and **33** from propolis.

Compound **1** was obtained as a colorless gum. The molecular formula of **1** was determined to be $C_{20}H_{21}O_6$ by HRFABMS. The IR spectrum of **1** indicated the presence of hydroxyl and carbonyl functions. The ^{1}H NMR spectrum of **1** in CD_3OD at 7.4−7.6 ppm indicated the presence of an aromatic ring system. The two aromatic proton signals at 5.94 and 5.96 ppm (H-6 and H-8), and two doublet signals at 5.41 and 5.87 ppm (H-2 and H-3) suggested that **1** has a pinobanksin moiety. Furthermore, several aliphatic signals at 0.58, 1.01, 1.43, and 2.31 ppm in the ^{1}H NMR spectrum of **1** were also observed. The ^{13}C NMR spectrum of **1** contained 18 carbon signals with two sets of overlapping peaks (**Table 1**). The signals in the ^{1}H and ^{13}C NMR spectra were assigned from the ^{1}H−^{1}H COSY, HSQC, and HMBC spectra. In the HMBC spectrum of **1**, the methine signal at 2.31 ppm and the methyl signal at 1.01 ppm were observed to correlate with a carbonyl carbon signal at 176.3 ppm (**Figure 5**), indicating **1** to be an isopentanoic acid ester. Configurations of the 2, 3 positions of **1** were determined to be 2R and 3R by comparison with the CD curve of pinobanksin derivatives in a previous report (*36*). However, configuration of the 2″ position is still uncertain. Consequently, the structure of **1** was determined to be 5,7-dihydroxy-3-isobutylflavanone, i.e., pinobanksin 3-(2-methyl)butyrate. Greenaway et al. (*48*, *49*) have reported that **1** might be present in the bud exudates of poplar tree (*Populus* species) by GC−MS analysis, but they did not isolate the compound and did not determine the full structure on the basis of NMR data. Thus, we isolated and identified this compound (**1**) for the first time (prior to the publication of ref *62*).



23: R=H
24: R=OCH_3



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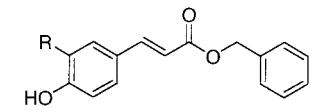
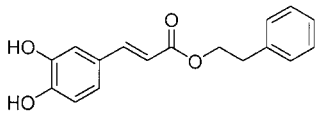


26: R=H
27: R=OCH_3



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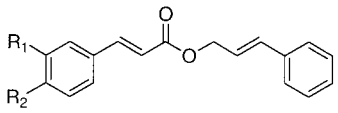
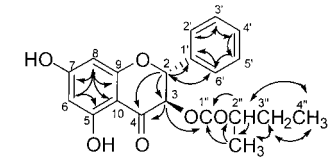


	R_1	R_2
29:	H	H
30:	H	OH
31:	OH	OH
32:	OH	OCH_3
33:	OCH_3	OCH_3

Figure 4. Structures of phenolic acid esters isolated from Uruguayan propolis.



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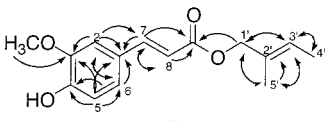

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Figure 5. Observed ^{1}H–^{13}C long-range couplings (H→C) for pinobanksin 3-(2-methyl)butyrate (**1**) and 2-methyl-2-butenyl ferulate (**24**).

Compound **2** was obtained as a colorless gum. The molecular formula of **2** was determined to be $C_{19}H_{18}O_6$ by HRFABMS. The IR spectrum of **2** indicated the presence of hydroxyl and carbonyl functions. The ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, and HSQC spectra of **2** showed the presence of pinobanksin moiety in the structure. In the HMBC spectrum of **2**, the proton signals at 2.49 and 5.90 ppm showed correlations with a carbonyl carbon signal at 175.4 ppm, indicating **2** to be an isobutyric acid ester. The course of the CD curve of **2** was similar to that of **1**. Thus, the structure of **2** was determined to be 5,7-dihydroxy-3-isobutylflavanone, i.e., pinobanksin 3-isobutyrate.

Compound **24** was obtained as a colorless gum. The molecular formula of **24** was determined to be $C_{15}H_{18}O_4$ by HR-

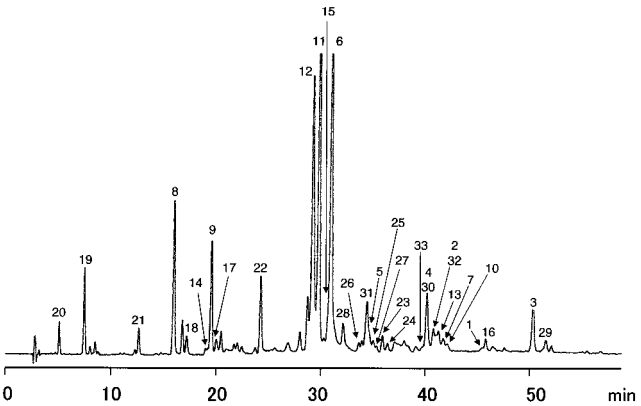

Figure 6. HPLC chromatogram of the ethanol extracts of Uruguayan propolis.

FABMS. The IR spectrum of **24** indicated the presence of hydroxyl and carbonyl functions. The ^{1}H and ^{13}C NMR of **24** were similar to those of **23** except for the signal of a methoxyl group. These NMR data indicated that one of two hydroxyl groups of **23** is a methoxyl group on **24**. The position of a methoxyl group in the structure of **24** was determined by $^{1}H-^{13}C$ long-range couplings of its HMBC spectrum. In the HMBC spectrum of **24**, the methyl proton signal at 3.92 ppm of the methoxyl group was observed to correlate with C-3 (**Figure 5**), indicating the methoxyl group to attach to C-3. Thus the structure of **24** was determined to be 2-methyl-2-butenyl ferulate. Although 2-methyl-2-butenyl isoferulate, a methoxyl group attached to C-4, has been already reported (*50*−*52*), this is the first isolation of **24**.

In this study, we isolated and identified thirty three compounds which are eighteen flavonoids (including two new compounds), four aromatic carboxylic acids, and eleven phenolic acid esters (including one new compound) from Uruguayan propolis. **Figure 6** shows the HPLC chromatogram of the ethanol extracts of Uruguayan propolis, and the compounds isolated in this study were assigned to each HPLC peak. Propolis from Europe and China contain many kinds of flavonoids and phenolic acid esters (*41*, *53*). In contrast, the major components in propolis of Brazilian origin were terpenoids and prenylated derivatives of *p*-coumaric acids, a difference that has been ascribed to the difference in the plant origin (*53*, *54*). It is generally accepted and chemically demonstrated that the bud exudates of poplar tree are the main source of propolis from Europe and China. Greenaway et al. (*44*) have reported that bud exudates of poplar include many kinds of flavonoids seen in propolis from Europe. However, Bankova et al. reported that *Baccharis* and *Araucaria* species are important sources of propolis in São Paulo state of Brazil (*55*, *56*). Midorikawa et al. reported that *Baccharis dracunculifolia* is an important source of propolis not only in São Paulo state but also in other states of Brazil (*57*). On the other hand, Park et al. (*58*, *59*) reported that Brazilian propolis could be classified into 12 groups by physicochemical methods and biological activity, and that the plant origins of propolis of southern, southeastern, and northeastern Brazil are poplar tree, *Hyptis divaricata*, and *Baccharis dracunculifolia*, respectively. We isolated many kinds of flavonoids and phenolic acid esters from Uruguayan propolis, and some of them were seen in propolis of Europe, China, and the southern Brazil origin. Therefore, as with propolis of these regions, one of the plant origins of Uruguayan propolis is assumed to be poplar tree. Bonvehí et al. (*31*) also reported that *Populus* sp. is one of the plant origins of Uruguayan propolis.

Uruguay is geographically located in the south of Brazil. The constituents of propolis from Uruguay were similar to not only the propolis from southern Brazil but also those of propolis from Europe and China as described above. In Uruguay, beekeepers use the ''native" bees and some strains of European honeybees (*60*). On the other hand, the honeybees bred in Brazil to collect propolis are mainly the Africanized honeybees (*54*). Africanized honeybees are the African bees (*Apis mellifera scutellata*) that were introduced into southeastern Brazil over 40 years and escaped to spread over other regions of Brazil (*61*). The European honeybees would tend to gather the bud exudates of poplar tree, whereas Africanized honeybees in Brazil would tend to gather the bud exudates of *Baccharis* species. Thus, it is considered that the constituents of propolis are also related to the difference of honeybees, although further studies on the chemical compositions of propolis and plants are needed for confirmation.

ACKNOWLEDGMENT

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